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Stem cell factor induces proliferation and differentiation of highly enriched murine hematopoietic cells

(stem cells/growth factors/hematopoiesis)

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ABSTRACT Recombinant rat stem cell factor (SCF) was studied for its ability to stimulate the growth of murine hematopoietic progenitor cells and to generate colony-forming cells (CFC) from highly enriched populations of hematopoietic cells. In serum-deprived cultures, SCF alone stimulated few colonies but interacted with a number of other hematopoietic growth factors, particularly interleukin 3, to promote colony formation. The most marked effect was on the generation of mixed-cell colonies. Hematopoietic cells were sorted into wheat-germ agglutinin-negative, monocyte-depleted, rhodamine 123 (Rh123)-bright or Rh123-dull cells. Historically, Rh123-bright cells are capable of short-term (<1 mo) marrow engraftment, whereas among Rh123-dull cells are cells capable of long-term marrow engraftment. Enriched cells (2.5×10^3) were placed into serum-deprived liquid cultures with various hematopoietic growth factors. Initially, the Rh123-bright and Rh123-dull cells had few CFC but, in the presence of interleukin 3 and SCF, Rh123-bright cells gave rise to >15,000 granulocyte/macrophage CFC, >1500 erythroid burst-forming cells, and >700 mixed-cell CFC by day 5. In contrast, Rh123-dull cells proliferated only in the presence of interleukin 3 and SCF, but total cell numbers rose to a peak of 18,000 by day 21, and one-third of the cells were CFC. Thus, SCF, in combination with other growth factors, can generate large numbers of CFC from pre-CFC and appears to act earlier than hematopoietic growth factors described to date.

An important goal of experimental hematology is understanding the factors that control proliferation and differentiation of pluripotent hematopoietic stem cells (PSC). In pursuit of this goal, several methods have been described recently to purify PSC from rodents and primates based on ability of the isolated cells to reconstitute hematopoiesis in appropriate recipients (1–4). PSC capable of long-term (>4 mo) marrow reconstitution (5–7) were separated from cells capable of short-term (<1 mo) repopulation. Cell function correlated with the degree to which such PSC are in the G_0 stage of the cell cycle (8). Because cell quiescence correlates with the mitochondrial activity of the cells, the two cell populations are separable based on differences in their ability to take up the dye rhodamine 123 (Rh123) (9).

Many hematopoietic growth factors have been identified that affect the proliferation and differentiation of hematopoietic progenitor cells (10). In contrast, little is known about the factors that control proliferation of PSC.

Rh123-dull and Rh123-bright populations can proliferate and colonize murine marrow. Although both cell populations die within 24 hr in culture without growth factors, they can induce a cobblestone area of hematopoiesis if seeded on marrow-stromal cells (11, 12). The factors produced by the marrow stroma responsible for PSC growth have not yet been identified. Rh123-bright cells can be induced into cell cycle by interleukin 3 (IL-3) (13, 14). Erythropoietin (Epo) (15), macrophage colony-stimulating factor (M-CSF), IL-1, and IL-6 (16, 17) synergize with IL-3 by synchronizing entry of these cells into the cell cycle (16). However, proliferation of these cells results predominantly in differentiation because the cells with marrow-repopulating ability are lost after only a 24-hr exposure to IL-3 (15), whereas the number of progenitor cells in the culture progressively increases, reaching a peak after 4 days (18). Because IL-3 has not been shown to be produced by stromal cells *in vivo* or *in vitro*, the identity of the growth factor that induces proliferation of Rh123-bright cells remains to be determined.

Recently stem-cell factor (SCF), the ligand for the c-*kit* gene product (19–22), has been isolated, and its gene has been cloned and expressed. SCF is expressed in a number of tissues during early development (23) and potentially is involved in the organization of multiple tissues, including hematopoiesis. We have studied the effect of recombinant rat SCF, alone and in combination with other hematopoietic growth factors, on the proliferation and differentiation of murine progenitor cells as well as highly enriched Rh123-bright and Rh123-dull cells. Although SCF had little colony-stimulating activity by itself, it interacted with all growth factors tested and, in conjunction with IL-3, generated large numbers of CFC from Rh123-bright and Rh123-dull cells. Thus, SCF is the most potent stimulus identified to date for generating many CFC from pre-CFC.

MATERIALS AND METHODS

Mice. B6C3J (The Jackson Laboratory) or CD1 (Charles River Breeding Laboratories) males (10–12 g) were used. Mice were provided with sterilized food and water ad libitum.

Growth Factors. Pure recombinant murine IL-3 and granulocyte/macrophage CSF (GM-CSF) were provided by J. J. Mermod (Glaxo). Pure recombinant human Epo and granulocyte CSF (G-CSF) and purified recombinant rat SCF (24) were provided by J. Egrie, L. Souza, and K.Z. (Amgen), respectively.

Each growth factor was used at a concentration that induced a maximal number of colonies in serum-deprived

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Abbreviations: CSF, colony-stimulating factor; SCF, stem cell factor; Rh123, rhodamine 123; CFC, colony-forming cells; PSC, pluripotent stem cells; IL, interleukin; Epo, erythropoietin; GM-, granulocyte/macrophage; M-CSF, macrophage CSF; FITC, fluorescein isothiocyanate; WGA, wheat germ agglutinin; G-CSF, granulocyte CSF; CFU-S, spleen colony-forming units; CFU-GM, GM colony-forming units; BFU-E, erythroid burst-forming cells; mAb, monoclonal antibody.

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cultures of total marrow. These concentrations were 100 units/ml for IL-3, 10 units/ml for GM-CSF, 1000 units/ml for G-CSF, 1.5 units/ml for Epo, and 100 ng/ml for SCF.

Enrichment of Hematopoietic Cells. Hematopoietic cells were enriched, as described (1, 25). Adult mouse marrow cells were collected by flushing the femurs with Hanks' balanced salt solution (GIBCO) buffered at pH 6.7 with Hepes (10 mM; Merck) and supplemented with penicillin (100 international units/ml) and streptomycin (0.1 mg/ml). Cells were filtered through a nylon sieve and then centrifuged (400 × *g* for 10 min at 4°C) in a discontinuous metrizamide (Nyegaard, Oslo) density gradient in the presence of wheat germ agglutinin (WGA) bound to fluorescein isothiocyanate (FITC; 1 μg/10 ml of metrizamide solution; Polysciences). The low-density cells were washed once and analyzed by using a light-activated cell sorter (FACS II; Becton Dickinson). Cells with medium and high WGA-FITC fluorescence, medium forward and low perpendicular light-scatter intensities, were sorted. Subsequently, WGA-FITC was removed from the cells by incubation with an isotonic solution of *N*-acetyl-D-glucosamine (0.2 mol/l; Polysciences). The cells were then labeled with the monoclonal antibody (mAb) 15-1.1 (26) directly conjugated with FITC and sorted again. The mAb 15-1.1-negative cells were stained with Rh123 (Eastman Kodak) and sorted again. Rh123-dull and -bright cells were separated on the basis of fluorescence. The effectiveness of separation was monitored by the spleen colony-forming assay (CFU-S). Preliminary experiments revealed a 1500-fold enrichment of day 12 CFU-S in the Rh123-bright population and a 250-fold increase in the Rh123-dull population (data not shown).

Cell Culture. Intact marrow cells or enriched cell populations were cultured either in semisolid medium to enumerate CFC, or 2.5×10^3 sorted cells were incubated in 0.5 ml of liquid culture for 2–21 days. In the latter case, the number of CFC in the culture was evaluated at different intervals by culturing an aliquot (20% of the volume) of cells from the suspension culture.

For CFC assays, 5×10^4 marrow cells were cultured in semisolid medium containing the following components in Iscove's modified Dulbecco's medium: methylcellulose (0.8%, final concentration), 2-mercaptoethanol (75 μM), and either 40% (vol/vol) fetal bovine serum (Hyclone) or a mixture of fetal bovine serum-replacing components—deionized bovine serum albumin (fraction V, Sigma) and bovine serum albumin-adsorbed cholesterol and soybean lecithin (Sigma; final concentration 200 μM for both), iron-saturated human transferrin (Behring; 9 μM), insulin (Sigma; 1.7 μM), nucleosides (10 μg/ml each), hemin (Sigma; 10 μM), sodium pyruvate (100 μM), and L-glutamine (2 mM), as reported (27, 28).

For liquid suspension cultures, methylcellulose was replaced by Iscove's modified Dulbecco's medium.

RESULTS

The Effect of SCF on CFC Growth. As shown in Fig. 1, increased concentrations of SCF interacted with Epo or G-CSF to promote the growth of erythroid bursts, granulocyte/macrophage (GM) colonies, or mixed-cell colonies. SCF, alone, supported the growth of few erythroid bursts; however, SCF synergized with Epo to provide more bursts than were obtainable with Epo plus spleen cell-conditioned medium (28). In contrast, SCF supported half the number of GM colonies found with G-CSF plus spleen cell-conditioned medium. SCF interacted with G-CSF in a dose-dependent fashion.

Although unable to promote mixed-cell colony growth by itself, SCF interacted with Epo to promote nearly as many mixed-cell colonies as seen with Epo plus spleen cell-conditioned medium.

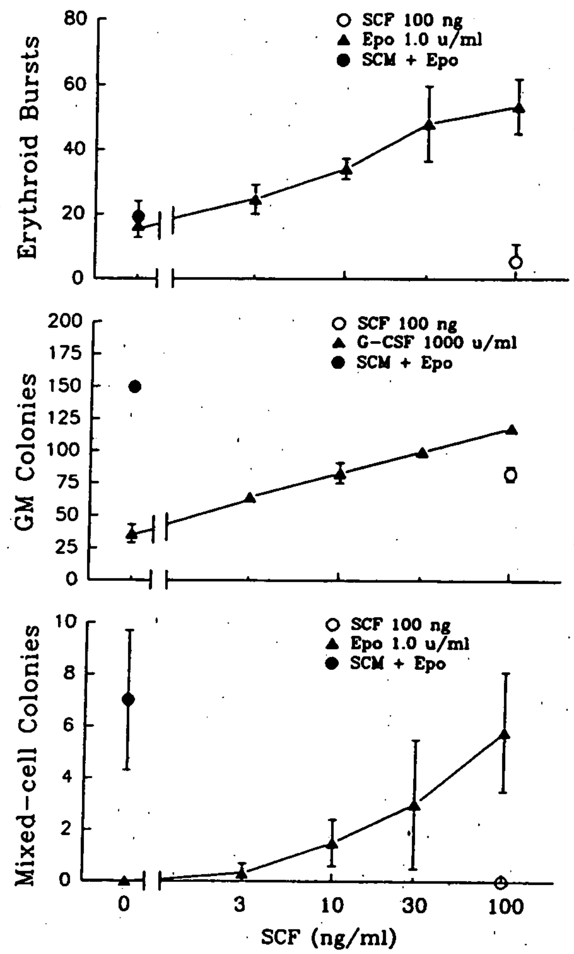

FIG. 1. Effect of increased concentrations of SCF on growth of erythroid bursts (*Top*), GM colonies (*Middle*), and mixed-cell colonies (*Bottom*) in serum-deprived cultures of unfractionated mouse marrow cells (5×10^4 cells per dish) in the presence of Epo or G-CSF. Number of colonies observed with 100 ng of SCF alone is also shown.

Fig. 2 shows the effect of SCF alone or in combination with other hematopoietic growth factors on colony formation by Rh123-bright cells. Erythroid bursts, GM colonies, and mixed-cell colonies were scored. SCF alone was incapable of supporting colony growth. IL-3, G-CSF, and GM-CSF, by themselves, supported only a few GM colonies. Epo alone supported no erythroid or mixed-cell colonies. In combinations, SCF with Epo gave rise to some erythroid bursts, whereas SCF, IL-3, and Epo, together, gave rise to 1–5 mixed-cell colonies, 50 GM colonies, and 5–10 pure erythroid bursts. SCF synergized with IL-3 to support GM colonies and synergized with G-CSF and GM-CSF to support the growth of GM colonies. Thus, SCF interacted with multiple hematopoietic growth factors to promote colony formation by a subpopulation of highly enriched murine hematopoietic cells.

Effect of SCF in Liquid Culture of Rh123-Bright and Rh123-Dull Cells. Fig. 3 shows the effect of SCF and IL-3, alone and in combination, on total cell numbers and CFC, over time, in serum-deprived liquid culture of Rh123-bright cells. The cell input was 2500 per well. After 2 days, the number of cells became virtually undetectable (Fig. 3*A*). Without growth factors, no cell proliferation occurred. In the presence of IL-3 alone, up to 30,000 cells were seen by day 5; this number declined by day 10. With SCF alone, several hundred cells

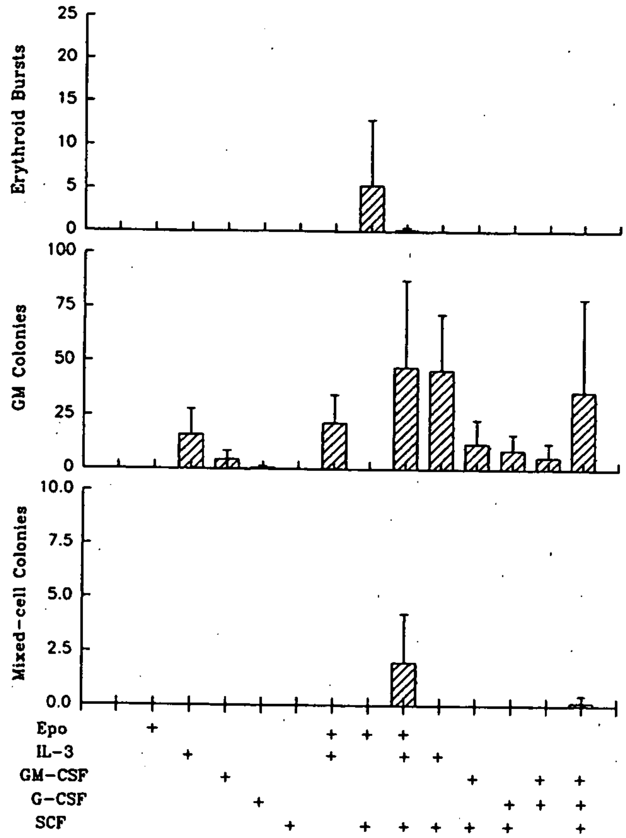

FIG. 2. Effect of SCF, alone or in combination with Epo, IL-3, GM-CSF, or G-CSF, on growth of erythroid bursts, GM colonies, and mixed-cell colonies (*Top*, *Middle*, or *Bottom*, respectively) from WGA$^+$/mAb 15-1.1$^-$/Rh123-bright marrow cells (500 cells per dish) in serum-deprived cultures. Data represent the mean (±SD) of three experiments performed in duplicate. Large SDs were seen due to the highly variable number of CFC detectable in primary cultures of Rh123-bright cells.

were present on day 5, but these became undetectable at day 10. IL-3 and SCF together, however, yielded nearly 200,000 cells by day 5, which declined to 100,000 by day 10.

Similar effects on CFC were seen (Fig. 3*B*). Few CFC were present in the Rh123-bright input cells. Without added growth factor, no CFC appeared. With IL-3, few GM colony-forming units (CFU-GM) were detected on day 2 but their numbers increased to ≈2000 by day 5. Some erythroid burst-forming cells (BFU-E) and mixed-cell CFC were detected by day 5. SCF alone gave rise to a small number of detectable BFU-E, GM-CFC, and mixed-cell CFC by day 5. Again, the combination of IL-3 and SCF was most dramatic in giving rise to 1500 mixed-cell CFC, nearly 15,000 GM-CFC, and over 700 BFU-E after 5 days in culture. CFC numbers declined by 10 days.

Subsequent experiments were done on day 5 cells to examine the interaction of SCF with other growth factors (Fig. 4). Again, without growth factors, cell numbers declined, and no CFC accumulated. SCF, G-CSF, and Epo, by themselves, promoted the accumulation of few CFC, and SCF and G-CSF maintained total cell numbers to a limited degree. In contrast, IL-3 and GM-CSF, by themselves, promoted considerable cell proliferation, and, for IL-3, the appearance of small numbers of BFU-E, GM-CFC, and mixed-cell CFC. GM-CSF is of particular interest in that it stimulated cell proliferation (≈100,000 from an input of 2500) but virtually no CFC. Most potent were SCF and IL-3; SCF and GM-CSF; and SCF, Epo, and IL-3. The addition of SCF

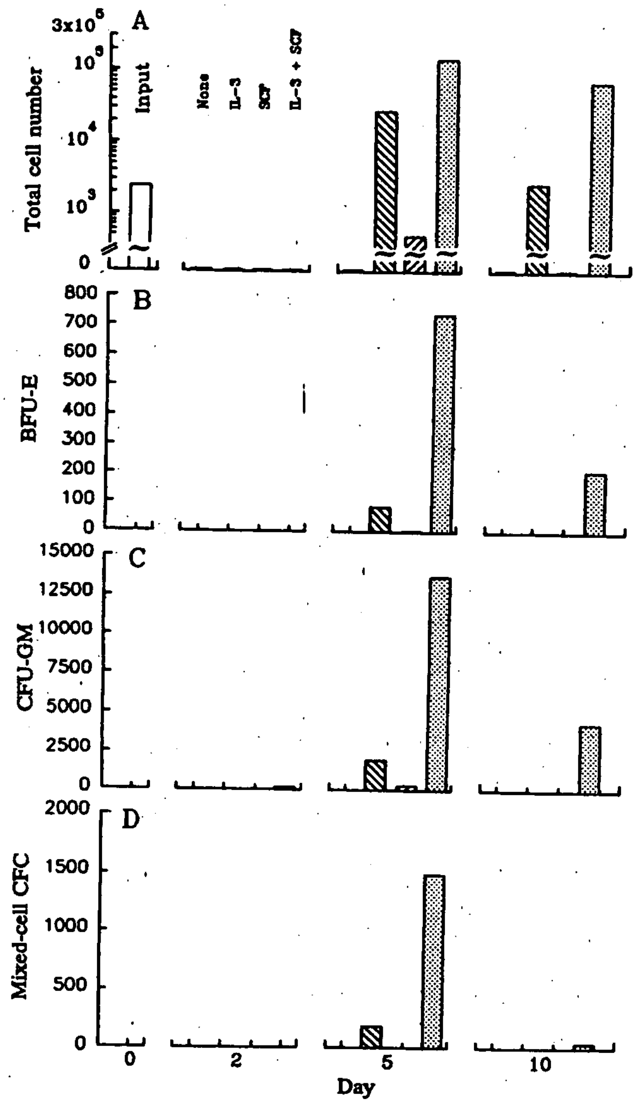

FIG. 3. Effect of SCF, alone or in combination with IL-3, on total cell numbers (*A*) or the number of BFU-E (*B*), CFU-GM (*C*), and mixed-cell CFC (*D*) arising in liquid culture of WGA$^+$/mAb 15-1.1$^-$/Rh123-bright marrow cells. Input cell values (day 0) and values after 2, 5, and 10 days are presented. Data represent the means of four separate experiments.

to the suspension culture resulted in much larger numbers of CFC of all types.

In experiments similar to those with Rh123-bright cells, 2500 Rh123-dull cells were placed into serum-deprived suspension culture. Results for 10 and 21 days of suspension culture are shown in Table 1, as no changes of consequence were seen during the first 5 days of culture. In these experiments, the Rh123-dull cells contained no detectable CFC at input. When no growth factors were added to the suspension cultures, no cells or CFC were recoverable. IL-3 alone, SCF alone, and the combination of G-CSF, GM-CSF, and IL-3 failed to sustain cell numbers or allow any accumulation of CFC. However, over a 10-day period, the combination of IL-3 and SCF not only maintained total cell numbers but also led to the appearance of BFU-E, GM-CFC, and mixed-cell CFC. These numbers increased further by day 21, at which point a nearly 8-fold amplification of total cell numbers was seen as well as more accumulation of BFU-E, GM-CFC, and mixed-cell CFC. By day 21, one-third of the cells were CFC.

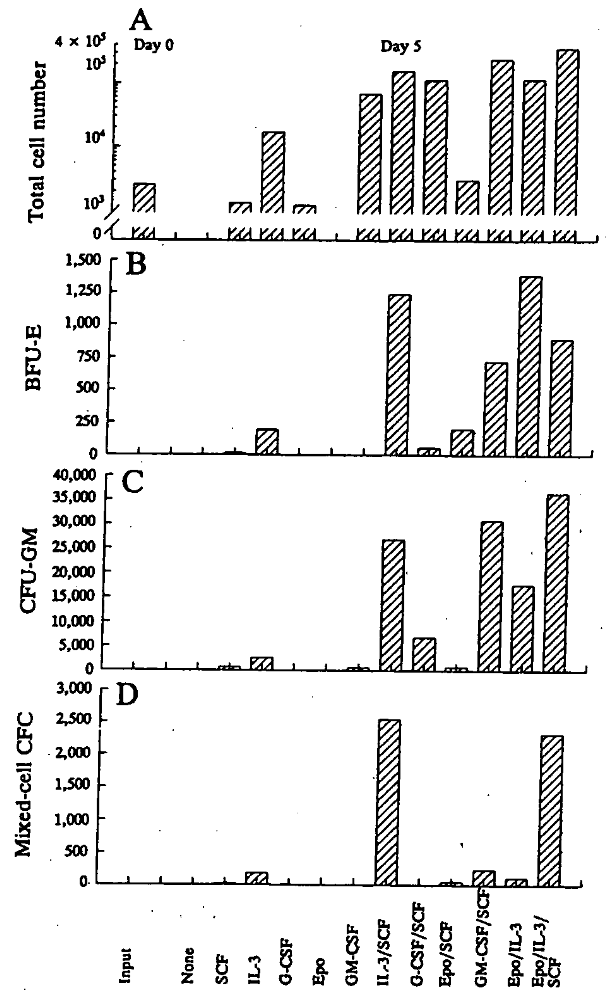

FIG. 4. Effect of SCF, alone and in combination with several hematopoietic growth factors, on proliferation and differentiation of WGA$^+$/mAb 15-1.1$^-$/Rh123-bright marrow cells after 5 days of liquid culture under serum-deprived conditions. Total cell number and numbers of BFU-E, CFU-GM, and mixed-cell CFC are shown in *A–D*, respectively. A representative experiment is shown.

DISCUSSION

SCF is an additional hematopoietic growth factor, the gene for which has recently been cloned and expressed (19–22, 24). SCF is encoded in the Steel locus and is the ligand for the c-*kit* tyrosine kinase receptor. Steel (*Sl/Sl^d*) mice have a number of developmental abnormalities, including altered coat color, lack of primordial germ cells, reduced support of hematopoietic stem cells, lack of mast cells, as well as macrocytic anemia (29). SCF is expressed differentially by a variety of tissues during murine embryogenesis and may have a role in development (23).

We have studied the effect of purified recombinant rat SCF on the *in vitro* proliferation and differentiation of adult murine hematopoietic progenitor cells and have examined the effect of SCF, alone or with other hematopoietic growth factors, on the proliferation and differentiation of highly enriched murine progenitor cells. These studies were designed to assess the range of progenitor cell activities of SCF and the range of potential interactions that SCF might have with other growth factors. The enrichment procedure chosen was designed to isolate populations of cells that had been shown previously to contain within them the cells responsible for short- or long-

Table 1. Effect of hematopoietic growth factors alone and in combination on proliferation and differentiation of Rh123-dull cells

Factor(s) added	Total cells/ml	BFU-E	GM-CFC	Mixed-cell CFC
Day 0	2,500	0	0	0
Day 10				
None	0	0	0	0
IL-3	0	0	0	0
SCF	0	0	0	0
IL-3 + SCF	2,770	25	982	31
G-CSF + GM-CSF + IL-3	0	0	0	0
Day 21				
None	0	0	0	0
IL-3	0	0	0	0
SCF	0	0	0	0
IL-3 + SCF	18,500	185	5,720	69
G-CSF + GM-CSF + IL-3	0	0	0	0

term marrow repopulation (Rh123-bright and Rh123-dull, respectively).

SCF alone was found to have little colony-stimulating activity in serum-deprived cultures. These results are similar to those of Broxmeyer *et al.* (30). Our findings are also similar to those made earlier for GM-CSF and IL-3 (15, 31) and are consistent with the thesis that early-acting factors require one or more later-acting factors for full colony growth to be observed. This hypothesis was borne out in studies that documented various patterns of interactions between SCF and IL-3, GM-CSF, G-CSF, and Epo.

In subsequent experiments, murine marrow cells were separated into Rh123-bright and Rh123-dull populations. Rh123-bright or Rh123-dull cells gave rise to few or no colonies when plated directly in semisolid medium with growth factors and without serum. Previous studies have demonstrated that both populations give rise to spleen colonies in the CFU-S assay. Rh123-bright cells contain both day 8 and day 12 CFU-S, whereas Rh123-dull cells contain predominantly day 12 CFU-S (11). Rh123-bright cells are incapable of long-term marrow reconstitution, suggesting that cells other than day 12 CFU-S, present among the Rh123-dull cells, are responsible for this function.

The results observed with the enriched cell populations confirmed that SCF can interact with a variety of hematopoietic growth factors and suggested that SCF was key to the accumulation of CFC over time. The results also demonstrated that the kinetics of response to SCF and other growth factors of Rh123-bright cells differed from the kinetics of the response of Rh123-dull cells. The peak response of Rh123-bright cells was on day 4 or 5 of suspension culture. For Rh123-dull cells, there was no early wave of proliferation and, under the conditions tested, the highest numbers of cells were seen at day 21. At this time, one-third of all cells that had accumulated were CFC of various classes, including mixed-cell CFC.

The kinetics of response to SCF in conjunction with IL-3 suggest that the more primitive cells are among the Rh123-dull population, in keeping with observations that this cell population is responsible for long-term marrow reconstitution. Furthermore, the results raise the possibility that SCF, alone or with one or another growth factor, can amplify the population giving rise to long-term marrow reconstitution.

The results also suggest other uses for SCF. If Rh123-dull cells can be activated into cell cycle by SCF, they provide a more prime target for gene-transfer experiments. Furthermore, if cells capable of long-term marrow reconstitution are amplified by SCF, strategies may be devised for the amplification of human stem cells *in vitro* and their storage for

eventual transplantation, because the gene for human SCF has also been cloned and expressed (24). The successful development of this methodology would simplify the transplantation process in a number of ways.

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1. Visser, J. W. M., Bauman, J. G. J., Mulder, A. H., Eliason, J. F. & de Leeuw, A. M. (1984) *J. Exp. Med.* **59**, 1576–1590.
2. Visser, J. W. M. & Van Bekkum, D. W. (1990) *Exp. Hematol. (N.Y.)* **18**, 248–256.
3. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. (1988) *Science* **241**, 58–62.
4. Berenson, R. J., Andrews, R. G. & Bensinger, W. I. (1988) *J. Clin. Invest.* **81**, 951–955.
5. Jones, R. J., Wagner, J. E., Celano, P., Zicha, M. S. & Sharkis, S. J. (1990) *Nature (London)* **347**, 188–189.
6. Spangrude, G. J. & Johnson, G. R. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7433–7437.
7. Micklem, H. S., Lennon, J. E., Ansell, J. D. & Gray, R. A. (1987) *Exp. Hematol. (N.Y.)* **15**, 251–257.
8. Hodgson, G. S. & Bradley, T. R. (1979) *Nature (London)* **281**, 381–382.
9. Johnson, L. V., Walsh, M. L., Bockus, B. J. & Chen, L. B. (1981) *J. Cell Biol.* **88**, 526–535.
10. Cross, M. & Dexter, T. M. (1991) *Cell* **64**, 271–280.
11. Visser, J. W. M., Hogeweg-Platenburg, M. G. C. & de Vries, P. (1990) in *The Biology of Hematopoiesis*, eds. Dainiak, N., Cronkite, E. P., McCaffrey, R. & Shadduck, R. (Wiley/Liss, New York), pp. 1–8.
12. Van der Sluijs, J. P., de Jong, J. P., Brons, N. H. C. & Ploemacher, R. E. (1990) *Exp. Hematol. (N.Y.)* **18**, 893–896.
13. Migliaccio, A. R. & Visser, J. W. M. (1986) *Exp. Hematol. (N.Y.)* **14**, 1043–1048.
14. Muller-Sieburg, C. E., Townsend, K., Weissman, I. L. & Rennick, D. (1988) *J. Exp. Med.* **167**, 1825–1840.
15. Migliaccio, G., Migliaccio, A. R. & Visser, J. W. M. (1988) *Blood* **72**, 944–951.
16. Dexter, T. M., Heyworth, C. M., Spooncer, E. & Ponting, I. (1990) *Philos. Trans. R. Soc. London Ser. B* **327**, 85–98.
17. Ikebuchi, K., Wong, G. G., Clark, S. C., Ihle, J. N., Hirai, Y. & Ogawa, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9035–9039.
18. Iscove, N. N. & Yan, X.-Q. (1990) *J. Immunol.* **145**, 190–195.
19. Williams, D. E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K., March, C. J., Park, L. S., Martin, U., Mochizuki, D. Y., Boswell, H. S., Burgess, G. S., Cosman, D. & Lyman, S. D. (1990) *Cell* **63**, 167–174.
20. Flanagan, J. G. & Leder, P. (1990) *Cell* **63**, 185–194.
21. Zsebo, K. M., Williams, D. A., Geissler, E. N., Broudy, V. C., Martin, F. H., Atkins, H. L., Hsu, R.-Y., Birkett, N. C., Okino, K. H., Murdock, D. C., Jacobsen, F. W., Langley, K. E., Smith, K. A., Takeishi, T., Cattanach, B. M., Galli, S. J. & Suggs, S. V. (1990) *Cell* **63**, 213–224.
22. Nocka, K., Buck, J., Levi, E. & Besmer, P. (1990) *EMBO J.* **9**, 3287–3294.
23. Matsui, Y., Zsebo, K. M. & Hogan, B. L. M. (1990) *Nature (London)* **347**, 667–669.
24. Martin, F. H., Suggs, S. V., Langley, K. E., Lu, H. S., Ting, J., Okino, K. H., Morris, C. F., McNiece, I. K., Jacobsen, F. W., Mendiaz, E. A., Birkett, N. C., Smith, K. A., Johnson, M. J., Parker, V. P., Flores, J. C., Patel, A. C., Fisher, E. F., Erjavec, H. O., Herrara, C. J., Wypych, J., Sachdev, R. K., Pope, J. A., Leslie, I., Wen, D., Lin, C.-H., Cupples, R. L. & Zsebo, K. M. (1990) *Cell* **63**, 203–211.
25. Visser, J. W. M. & de Vries, P. (1988) *Blood Cells* **14**, 369–384.
26. Visser, J. W. M. & de Vries, P. (1990) *Methods Cell Biol.* **33**, 451–468.
27. Migliaccio, G. & Migliaccio, A. R. (1987) *Br. J. Haematol.* **67**, 129–133.
28. Migliaccio, G. & Migliaccio, A. R. (1987) *Ann. N.Y. Acad. Sci.* **511**, 39–49.
29. Sarvella, P. A. & Russell, L. B. (1956) *J. Hered.* **47**, 123–128.
30. Broxmeyer, H. E., Hangoc, G., Cooper, S., Anderson, D., Cosman, D., Lyman, S. D. & Williams, D. E. (1991) *Exp. Hematol. (N.Y.)* **19**, 143–146.
31. Migliaccio, G., Migliaccio, A. R., Kaushansky, K. & Adamson, J. W. (1989) *Exp. Hematol. (N.Y.)* **17**, 110–115.

Long-Term Generation of Human Mast Cells in Serum-Free Cultures of CD34$^+$ Cord Blood Cells Stimulated With Stem Cell Factor and Interleukin-3

By Brigitte Durand, Giovanni Migliaccio, Nelson S. Yee, Keith Eddleman, Tellervo Huima-Byron, Anna Rita Migliaccio, and John W. Adamson

The generation of murine mast cells is supported by several cytokines, and mast cell lines are frequently established in long-term cultures of normal murine marrow cells. In contrast, growth of human mast cells was initially dependent on coculture with murine fibroblasts. The growth factor produced by murine fibroblasts and required to observe differentiation of human mast cells is attributable in part to stem cell factor (SCF). However, other factors are likely involved. We have previously shown that the combination of SCF and interleukin-3 (IL-3) efficiently sustains proliferation and differentiation of colony-forming cells (CFCs) from pre-CFC enriched from human umbilical cord blood by CD34$^+$ selection. With periodic medium changes and the addition of fresh growth factors, five consecutive cultures of different cord blood samples gave rise to differentiated cells and CFCs for more than 2 months. Although differentiated cells continued to be generated for more than 5 months, CFCs were no longer detectable by day 50 of culture. The cells have the morphology of immature mast cells, are Toluidine blue positive, are karyotypically normal, are CD33$^+$, CD34$^-$, CD45$^+$, c-kit$^-$, and c-fms$^-$, and die in the absence of either SCF or IL-3. These cells do not form colonies in semisolid culture and are propagated in liquid culture stimulated with SCF and IL-3 at a seeding concentration of no less than 10^4 cells/mL. At refeedings, the cultures contain a high number (>50%) of dead cells and have a doubling time ranging from 5 to 12 days. This suggests that subsets of the cell population die because of a requirement for a growth factor other than SCF or IL-3. These results indicate that the combination of cord blood progenitor and stem cells, plus a cocktail of growth factors including SCF and IL-3, is capable with high efficiency of giving rise in serum-deprived culture to human mast cells that behave like factor-dependent cell lines. These cells may represent a useful tool for studies of human mast cell differentiation and leukemia.

PROLIFERATION OF murine mast cells is sustained by several cytokines including interleukin-3 (IL-3), IL-4, IL-9, and IL-10[1-4], as well as stem cell factor (SCF),[5-8] which is also termed mast cell growth factor[9] or kit ligand.[5] Furthermore, in long-term cultures of normal murine marrow cells, cell lines have frequently been established based on their dependence on IL-3 for growth.[10,11,12] Although they retain the capacity to differentiate along other hematopoietic lineages when stimulated with appropriate growth factors,[13,14,15] most of these cell lines have a mast cell phenotype.[10] The cells are karyotypically normal by light microscopy and they do not induce tumors when injected into syngeneic recipients. The molecular events that result in immortalization of these cell lines are unknown.

In contrast, several human cytokines, including IL-3, have failed to sustain proliferation of human mast cells and, to date, no cell lines have been established from normal human long-term marrow cultures. However, human stromal cells supporting long-term hematopoiesis are less efficient than their murine counterparts.[16] In vitro, growth and differentiation of human mast cells have been reported in cocultures of mononuclear cord blood cells and Swiss albino/3T3 fibroblasts.[17] The growth factor produced by the murine fibroblasts and responsible, at least in part, for proliferation and differentiation of the human mast cells, recently has been identified as SCF,[18] the ligand for the receptor encoded by the proto-oncogene c-kit.[5,9,19] In fact, several independent reports have shown human mast cell differentiation from unfractionated cord blood mononuclear cells[18] or adult blood and marrow[20] in serum-supplemented cultures stimulated with SCF. Cells with a mast cell phenotype were first detected after 4 weeks of culture[18,20] and became the predominant cell population by week 13. The cultures were not maintained beyond that point. However, the establishment of rat mast cell lines has been reported in cultures stimulated with SCF.[21]

We have previously shown that the combination of SCF and IL-3[22,23] is at least as efficient as a stromal layer[24] in sustaining the proliferation and differentiation of colony-forming cells (CFCs) from pre-CFC in serum-deprived liquid culture. The target cells for our studies were CD34$^+$ cells isolated from human umbilical cord blood. In this report, we describe the establishment of long-term cultures of human mast cells from normal human CD34$^+$ cord blood cells in stroma-free suspension culture stimulated with SCF and IL-3 under serum-deprived conditions.

MATERIALS AND METHODS

Collection and separation of cord blood cells. Umbilical cord blood samples were collected at the New York Hospital-Cornell

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University Medical Center under protocols approved by the Institutional Review Boards of both the New York Hospital–Cornell University Medical Center and New York Blood Center.

The cells were first separated by centrifugation over a density gradient (1.077 g/mL; Ficoll-Hypaque; Pharmacia, Uppsala, Sweden). The light-density cells were then depleted of adherent cells and T-lymphocytes by a modification[25] of the soybean agglutination (SBA) method of Reisner et al.[26] The SBA$^-$ cells were then adhered to flasks coated with anti-CD34 antibody (Applied Immune Sciences, Menlo Park, CA). Nonadherent cells were removed and then adherent cells (defined as CD34$^+$) were harvested by mechanical agitation of the flask as described.[23] Alternatively, CD34$^+$ cells were separated directly from the light-density cell fraction by affinity chromatography with the Ceprate device (Cellpro, Bothell, WA), as described by the manufacturer. The frequencies of CFC and of CD34$^+$ cells in the CD34$^+$ cell populations purified according to the two techniques were subsequently analyzed in semisolid cultures (see below), or reanalyzed by flow cytometry after staining with a CD34-specific antibody (Gen Trak, Plymouth Meeting, PA). The frequencies of CD34$^+$ cells as determined by fluorescence-activated cell-sorting analysis were 3% or 30% for the cells purified by panning or by affinity chromatography, respectively, and were comparable with the frequencies of CFC detected in the two populations. Despite the differences in the frequencies of CD34$^+$ cells and CFC in cells purified by either of the two techniques, similar results were obtained in liquid culture.

Hematopoietic growth factors. The purified recombinant human hematopoietic growth factors used included erythropoietin (Epo), granulocyte colony-stimulating factor (G-CSF), SCF (all from Amgen, Thousand Oaks, CA), and IL-3 (Genetics Institute, Cambridge, MA). G-CSF, IL-3, and SCF were used at concentrations that induced the optimal response in fetal bovine serum (FBS)-deprived cultures of human marrow cells.[25,27] These concentrations are 2×10^{-10} mol/L of G-CSF, 2×10^{-10} mol/L of IL-3, 100 ng SCF/mL, and 1.5 U Epo/mL per culture.

Establishment of mast cell cultures from CD34$^+$ cord blood cells. Harvested CD34$^+$ cord blood cells (2.5×10^4 purified cells/flask) were incubated at 37°C in liquid culture under serum-deprived conditions[28] in the presence of recombinant human IL-3 (2×10^{-10} mol/L: Genetics Institute, Cambridge, MA) and recombinant human SCF (100 ng/mL; Amgen, Thousand Oaks, CA). The concentrations of human IL-3 and SCF used in this paper were previously shown to induce optimal proliferation of human progenitors[22,23] and mast cells.[6] The serum-deprived culture medium was composed of Iscove's modified Dulbecco's medium (IMDM) supplemented with β-mercaptoethanol (7.5×10^{-5} mol/L), antibiotics (100 U of penicillin, 250 ng of amphotericin B, and 100 μg of streptomycin), deionized bovine serum albumin (BSA; 2×10^{-4} mol/L), BSA-adsorbed cholesterol (4 μg/mL), and soybean lecithin (12 μg/mL), iron-saturated human transferrin (5×10^{-7} mol/L), insulin (1.7×10^{-6} mol/L), nucleosides (10 μg/mL each), inorganic salts, sodium pyruvate (10^{-4} mol/L), and L-glutamine (2×10^{-3} mol/L). All the chemicals were obtained from Sigma Chemical Co (St Louis, MO). Cell growth was monitored periodically with an inverted microscope. When the cell concentration in the flasks appeared to reach more than 0.5×10^6/mL, the cultures were demi-depopulated by replacing 50% of the medium with fresh medium and growth factors.[22] The removed cells were counted and immunophenotyped and their content of CFCs was evaluated in semisolid cultures.

Colony assays. The CFC content of the harvested cells was evaluated in a standard methylcellulose assay. Briefly, each 1-mL dish contained FBS (Hyclone, Logan, UT; 30% vol/vol), BSA (0.9%, wt/vol), β-mercaptoethanol (7.5×10^{-5} mol/L), antibiotics (100 U of penicillin, 250 ng of amphotericin B and 100 μg of streptomycin), and methylcellulose (0.8%, wt/vol, final concentration) in IMDM.

Table 1. Antigenic Markers of Long-term Cultures Derived From CD34$^+$ Cord Blood Cells

	Mast Cell Cultures No. 38	Mast Cell Cultures No. 41	Specificity
CD34 (Gen Trak Inc, Plymouth Meeting, MA)	--	--	pre-CFCs, CFCs
CD33 (Gen Trak Inc)	+	+	gp 67, CFCs, monocytes, mast cells
HLA-DR (Gen Trak Inc)	–	–	B cells, monocytes, activated T cells
CD3 (Gen Trak Inc)	–	–	T-cell receptor
CD45 (Gen Trak Inc)	±	±	Leukocyte common antigen
CD14 (Gen Trak Inc)	–	–	Monocytes, macrophages, granulocytes
CD16 (Gen Trak Inc)	–	–	NK cells, granulocytes
CD19 (Gen Trak Inc)	–	–	B cells
CD 42b (Amac Inc, Westbrooke, ME)	–	–	Platelet gpIb
CD 56 (Gen Trak Inc)	–	–	NK cells
CD W64 (Harlan Inc, Indianapolis, IN)	–	–	Monocytes
c-kit (Dr A. Ulrich or Amac Inc)	+	+	CFCs, mast cells
c-fms/CSF-1 receptor (Oncogene Science Inc, Manhasset, NY)	–	–	Monocytes

Abbreviation: NK, natural killer.

Colony growth was stimulated with combinations of growth factors at appropriate concentrations, including Epo (1.5 U/mL), IL-3 (2×10^{-10} mol/L), and SCF (100 ng/mL) for erythroid burst-forming cell (BFU-E) growth and mixed-cell CFC growth and G-CSF (2×10^{-10} mol/L), IL-3 (2×10^{-10} mol/L), and SCF (100 ng/mL) for granulocyte-macrophage CFC (GM-CFC) growth. Colonies were identified by their characteristic features after 12 to 14 days in culture and enumerated as described.[25]

Characterization of the cell cultures. Cell-surface phenotype was determined by cytofluorimetric analysis on FACScan (Becton Dickinson, Mountain View, CA) of cells incubated with several antibodies specific for antigens expressed on hematopoietic cells. The source of the antibodies is specified in Table 1. Cytochemical analysis was performed with specific kits provided by Sigma or with Toluidine blue (1% wt/vol in McIlvaine buffer, pH 4.0). For electron microscopy studies, the cells were fixed in suspension with phosphate-buffered 3% glutaraldehyde, osmicated, and embedded in PolyBed 812. Thin sections were examined with a Philips EM 410 electron microscope. Karyotypic analysis was performed in the Laboratory of Human Genetics of the Lindsley F. Kimball Research Institute by Dr James German.

RNA preparation and Northern blot analysis. RNA was extracted with phenol-chloroform from acid guanidinium-isothiocyanate cell lysates.[29] RNA was size-fractionated by electrophoresis on agarose (1%) gel under denaturing conditions and blotted onto nylon membranes (Bio-Rad Laboratories, Richmond, CA) that were subsequently hybridized with the human c-kit (American Tissue Culture Collection depository), myeloperoxidase (a gift of Dr G. Rovera, Wistar Institute, Philadelphia, PA), β-globin, the α chain of the F_cE receptor (Dr C. Wood, Genetics Institute, Cambridge, MA), or glyceraldehyde-3-phosphate dehydrogenase (G3PD)[30] probe, as indicated. Each probe was radiolabeled by random oligonucleotide

priming (Amersham International, Amersham, UK) to a specific activity of 4 to 8 × 10[8] dpm/mg. After probing, the membranes were washed as recommended by the manufacturer and exposed for appropriate lengths of time with X-Omat film (Sigma) in cassettes for autoradiography (Amersham).

DNA preparation and Southern blot analysis. The possibility of Epstein-Barr virus (EBV) infection was investigated by Southern analysis. High molecular-weight genomic DNA was prepared by the procedure of Herrman and Frischauf.[31] DNA (10 mg) was digested with *Pst* 1 and *Hin*dIII (New England Biolabs, Beverly, MA), separated by electrophoresis on 0.8% agarose gel, and transferred to a nylon membrane. Membranes were baked at 80°C for 30 minutes in a vacuum oven and hybridized with cDNA radiolabeled by random oligonucleotide priming (Amersham). The probe represented a fragment of the virus genome (p 107.5) and was kindly provided by Dr Riccardo Dalla Favera (Columbia University, New York, NY).

RESULTS

Long-term generation of cord blood cells. The growth pattern of CD34$^+$ cord blood cells in liquid culture stimulated with IL-3 and SCF is shown in Fig 1. During the first 2 to 3 months, large numbers of differentiated cells including erythroid as well as myelomonocytic cells[23] and CFCs of all types (BFU-E, GM-CFC, and mixed-cell CFC)[23] were generated. CFC were no longer detectable after 50 days of culture, but differentiated cells continued to accumulate. The morphology of these cells became more uniform over time

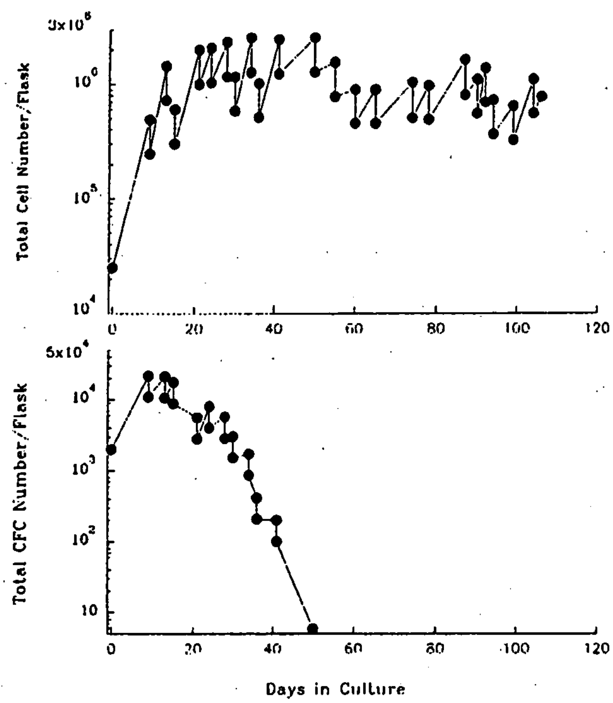

Fig 1. Total cell number (top) and progenitors of all types (bottom) in a typical long-term culture of CD34$^+$ cord blood cells stimulated with SCF and IL-3. At each time point, the cultures were demi-depopulated as indicated by the double values connected with a vertical line. The values have not been corrected for demi-depopulation. The number of CFCs decreased to levels below detection by day 50, whereas large numbers of cells continued to be generated throughout the culture period.

(Fig 2A). Beyond 3 months, cell proliferation was maintained with medium changes and the addition of fresh growth factors every 3 to 4 days.

The different mast cell cultures obtained are summarized in Table 2. The oldest cultures are now 5 to 8 months old and the cells have a doubling time ranging from 5 to 12 days. Furthermore, if cryopreserved, these cells can be thawed and cultured again for another prolonged period of time (>4 months) with no change in morphology or growth parameters; therefore, these cells behave like growth-factor–dependent cell lines. The long doubling time results from the fact that the cultures contain a high number (>50%) of trypan blue-positive (nonviable) cells.

The proliferation of these cells is dependent on the presence of SCF in combination with IL-3. Cells cultured in the absence of growth factor or in the presence of either SCF or IL-3 alone died within a few days. The growth of these cultures is also cell concentration-dependent since cultures were lost when initiated with a cell concentration less than 10[4]/mL. No proliferation was observed if FBS (up to 20% vol/vol) was added to the liquid cultures. The cells failed to proliferate in semisolid culture at any cell concentration—even in FBS-deprived conditions.

Morphology, cell surface phenotype, and cytochemical analysis. To identify and characterize the cells generated in culture, two of the long-term cultures, #38 and #41, were examined for cell morphology, immunophenotype, and cytochemical markers at months 7 and 4, respectively. As observed by light microscopy, May-Grünwald-Giemsa–stained cells were mononuclear, filled with cytoplasmic granules, and had a mast cell-like morphology (Fig 2A). Cell-surface expression of CD33 and c-kit further indicated that the cultures contained mast cells (Table 1 and Fig 3). These findings were consistent with the detection of metachromatic granules in the cytoplasm of Toluidine blue-stained cells and more than 90% of all viable cells were Toluidine blue-positive (Fig 2B and Table 3). However, although the cells did not express myeloperoxidase or β-globin (Fig 4), they were positive for markers specific for other cell types, including tartaric acid-sensitive acid phosphatase, tartaric acid-resistant acid phosphatase, and naphthol AS-D chloroacetate esterase (Fig 5 and Table 3). This raised the possibility that the in vitro-derived mast cells are immature. In agreement with this, a representative cell from the generated cultures exhibited ultrastructural features of immature mast cells as shown by electron microscopy.[32] The micrograph in Fig 6 showed that, except for small indentations, the nucleus was oval and unsegmented, it displayed a dispersed chromatin pattern, and there were numerous cytoplasmic granules that had a heterogeneous content. Further indication that the mast cells were immature came from the fact that the granules were negative for safranin staining and the cells did not express detectable levels of the α chain of the high affinity F_cE receptor (not shown), another marker of mature mast cells.[33]

Expression of c-kit protein/RNA and downmodulation by SCF. To determine the homogeneity of the long-term cultured mast cells with regard to cell surface expression of c-kit receptor, a human c-kit–specific monoclonal antibody was used for immunofluorescence analysis and it is reason-

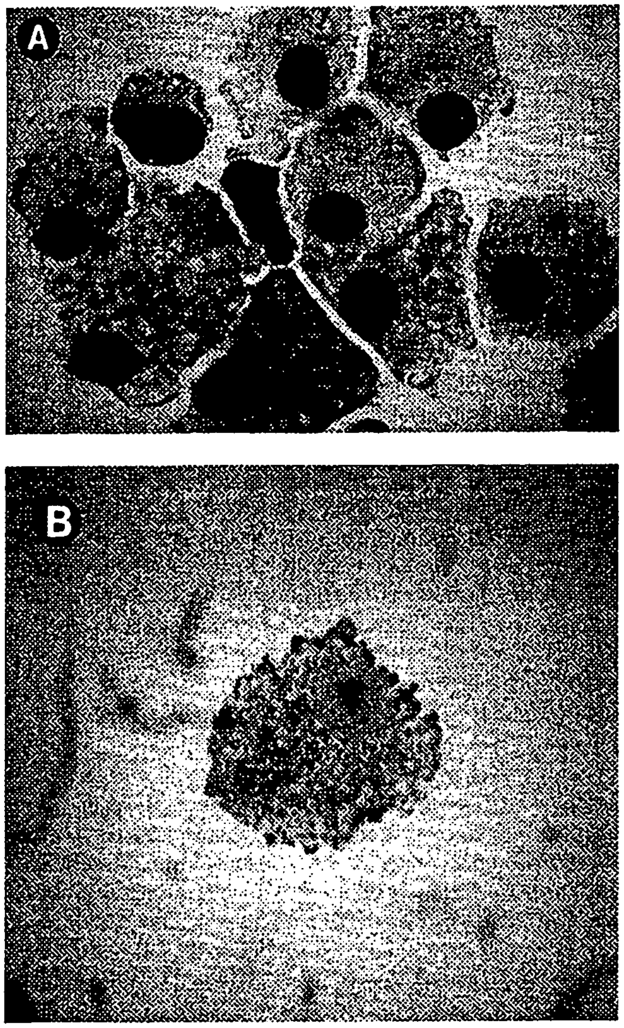

Fig 2. May Grünwald Giemsa (A) and Toluidine blue (B) staining of the cells recovered after 4 months in liquid culture of CD34⁺ cord blood cells stimulated with SCF and IL-3 (original magnification × 100).

able to assume that the fluorescence intensity correlates with the level of c-kit protein. As shown in Fig 3, almost all the cells expressed c-kit protein on the cell surface with a unimodal distribution. This indicates that the mast cell culture generated represents a relatively homogeneous population.

Because SCF and IL-3 are constantly present in the medium, they may influence the steady-state level of c-kit protein on these cells. Removal of both SCF and IL-3 from the culture medium for 6 hours elevated the mean fluorescence intensity (MFI) on the cell surface twofold (79.8 ± 13.6 *v* 39.1 ± 4.4, $P < .01$). The increase in the level of c-kit protein

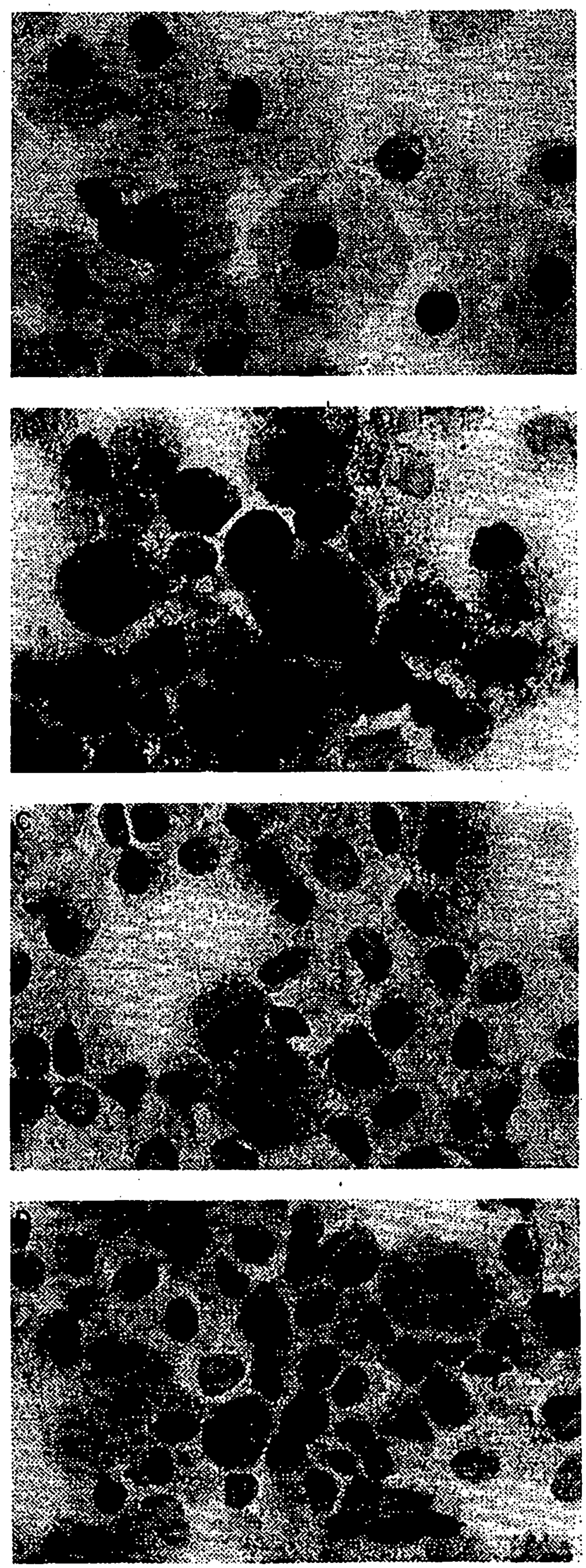

Fig 5. Cytochemical staining of the cells recovered after 4 months of liquid culture in the presence of SCF and IL-3 (original magnification × 100). (A) Alkaline phosphatase; (B) acid phosphatase; (C) tartaric acid-resistant acid phosphatase; and (D) naphthol AS-D chloroacetate esterase are shown.

Table 2. Summary of Long-term Mast Cell Cultures Obtained to Date

Cell Line	Time in Culture	CFCs Detected Until Day	Doubling Time	Status
27	5 mo	ND	—	Cryopreserved
37	3 mo	55	—	Cryopreserved at day 87
38	7-8 mo	70	6-12 d	Still growing
41	5-6 mo	70	4-5 d	Still growing
45	2 mo	ND	—	Still growing

Abbreviation: ND, not determined.

Table 3. Cytochemical Markers of Cultures Derived From CD34⁺ Cord Blood Cells

	CD34⁺ Cord Blood Cells	Mast Cell Cultures		Specificity
		No. 38	No. 41	
Alkaline phosphatase	++	−	−	Neutrophils
Tartaric acid-sensitive acid phosphatase	±	+	+	Most leukocytes
Tartaric acid-resistant acid phosphatase	−	+	+	Some lymphocytes, monocytes, epithelial cells
Naphthol AS-D chloroacetate esterase	++	+	+	Granulocytes, monocytes (weak)
α-Naphtyl acetate esterase	−	±	±	Monocytes-macrophages
Toluidine blue	ND	ND	+	Mast cells

Abbreviation: ND, not determined.

was rapid and dependent on the duration of deprivation of the growth factors, such that an increase in relative intensity of 20% was observed as early as 1 hour after withdrawal of SCF from the culture. The c-kit–specific fluorescence intensity (40 ± 5) for cells maintained in the presence of SCF alone for 6 hours was similar to that for cells incubated with both SCF and IL-3 (39.1 ± 4.4), indicating that SCF is the predominant modulator of c-kit expression. This is further supported by the finding that the increases in c-kit–specific fluorescence intensities were similar whether the cells were maintained in the presence of IL-3 alone or in the absence of both SCF and IL-3. The upregulation of c-kit receptor level in these human mast cells upon deprivation of SCF is in agreement with reports that kit ligand downregulates the cell surface expression of c-kit protein on murine bone marrow–derived mast cells by accelerating receptor internalization and receptor ubiquitination/degradation.[34,35]

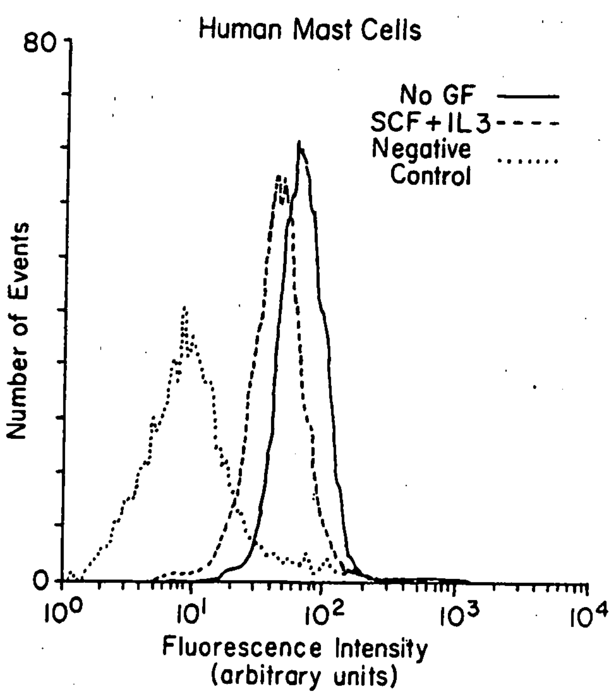

Fig 3. Flow-cytometric analysis of expression of c-kit protein on the surface of the human long-term mast cell culture CB 38 growing in the presence of SCF + IL-3 (---) or starved of growth factors for 6 hours (—). An antibody unrelated to c-kit was used as negative control and included for comparison (. . . .). The results shown are representative of three independent experiments. The mast cells expressed c-kit on the cell surface with a unimodal distribution peaking at 39.1 ± 4.4 arbitrary fluorescence units. Growth factor starvation for 6 hours doubled the MFI expressed by the cells (mean ± SD of the fluorescence peak, 79.8 ± 13.6, $P < .01$).

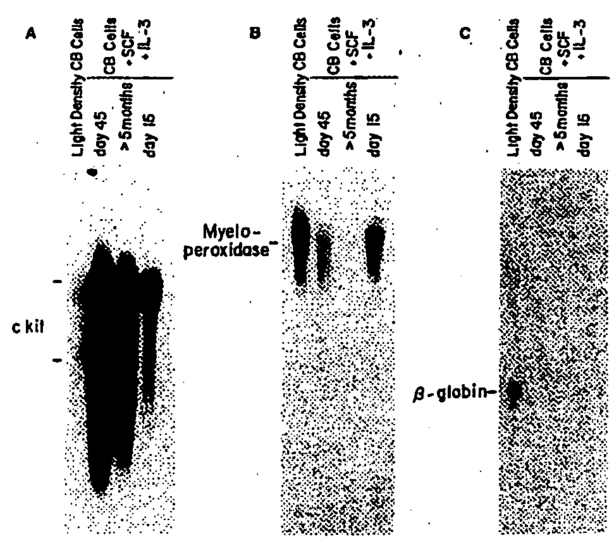

Fig 4. Northern analysis of the expression of c-kit (A), myeloperoxidase (B), and β-globin (C) in the light-density fraction of cord blood cells (lane 1) or in CD34⁺ cord blood cells growing in liquid culture for 45 days (lane 2) or more than 5 months (lane 3) in the presence of SCF + IL-3. Day 15 cells (lane 4) were obtained from colonies growing in semisolid cultures of cord blood cells in the presence of SCF, IL-3, GM-CSF, G-CSF, and Epo; mixed-cell colonies predominated in these cultures. Because of the low number (≅10^6 cells) of purified CD34⁺ cord blood cells that could be obtained and cultured (≅2.5×10^4 cells/flask), it was not possible to determine the expression of these genes at the outset of culture. mRNAs of the appropriate sizes were detected for c-kit, myeloperoxidase, and β-globin (although barely detectable after overnight exposure) in light-density cord blood cells and in CD34⁺ cells cultured for 15 days in semisolid medium. Expression of β-globin and myeloperoxidase was undetectable after day 45 or after 5 months of culture, respectively, whereas c-kit was expressed at high levels throughout the culture period investigated. Equivalent levels of expression of G3PD were detectable in all the samples (not shown). Exposure times were 16 hours for c-kit and β-globin and 4 hours for myeloperoxidase.

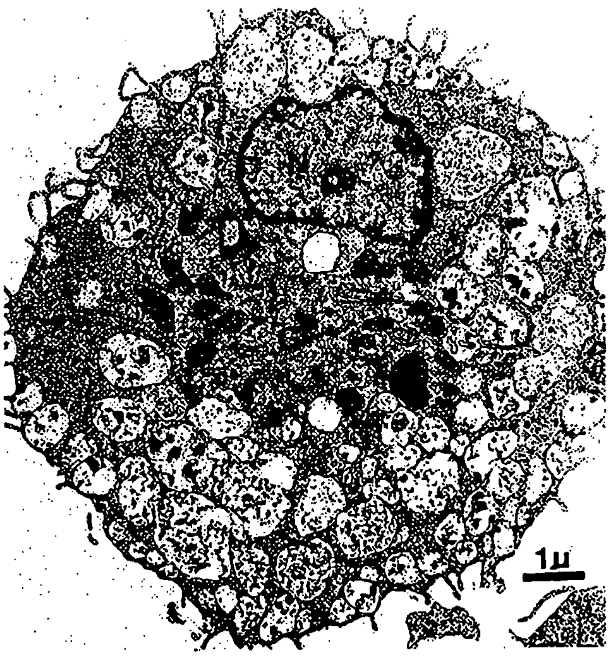

Fig 6. Electron-microscopic analysis of a cell generated in culture after 4 months. A representative cell is shown (original magnification × 10,000). The cell surface is smooth with a varied number of short microvilli. The golgi area (G) is well developed and the mitochondria are very few in number. Numerous secretory granules have a thin membrane with heterogeneous contents and some membranous structures. N indicates the nucleus.

Two c-kit RNA species were detected in the long-term cultures of cord blood cells (Fig 4A). Yarden et al[36] reported that a single c-kit transcript of 5 kb was detected in Northern blots with human placental poly(A)$^+$ RNA. It is unclear whether the lower–molecular-weight RNA observed here is c-kit mRNA or if it represents an alternatively spliced c-kit RNA. However, both of these RNA species increased proportionately over time in the cultures with SCF and IL-3.

Karyotypic analysis. Culture 37 was diploid, with the karyotype 46,XY. High-resolution G-banding of 20 cells showed the absence of gross deletions and rearrangements.

Analysis for the presence of EBV in cells. Southern analysis of the cell lines showed no evidence for EBV infection.

DISCUSSION

Previously, we[22,23] and others [37] have reported on the long-term suspension culture of CD34$^+$ cord blood mononuclear cells. We showed the potential for such suspension cultures to generate large numbers of CFCs from pre-CFCs as well as differentiated cells of a variety of lineages, including macrophages, mast cells, neutrophils, and, in the presence of Epo, erythroblasts.

SCF and other growth factors, such as Epo or G-CSF, gave rise to short-term (up to 15 days) cultures containing large numbers of erythroblasts or neutrophils, respectively, whereas long-term (up to 1 month) maintenance of these cultures under serum-deprived conditions requires the presence of SCF and IL-3. We have now extended these studies to show that with refeeding of serum-deprived cultures and the addition of fresh growth factors, including SCF and IL-3, the culture can be maintained indefinitely. However, the morphology of the cells generated in culture changes over time. Whereas cells expressing β-globin and myeloperoxidase prevail at early time points (day 15), cells expressing myeloperoxidase prevail from day 15 to the end of the third month. At this point, the cell population generated in culture became homogeneous in morphology and myeloperoxidase negative, expressed high levels of c-kit, and had the ultrastructural morphology consistent with that of immature mast cells. In our study, continuously growing cultures have been maintained for up to 8 months in the presence of SCF and IL-3 and in the absence of a source of serum. Interestingly, the colony-forming ability of the cells was lost by the 50th day of culture, but differentiated cells continued to accumulate. The cells could be frozen and thawed repeatedly without change in morphology or growth characteristics. Because progenitor cells were not detectable by this time and the culture could be propagated with as few as 10^4 cells/mL, these mast cells have the potential to self-replicate and, therefore, behave as cell lines. However, we did not formally prove these are cell lines because we have been unable to clone them. They would not grow in semisolid culture or in liquid culture under limiting dilution conditions. It is possible that normal human mast cells might retain a limited self-replication potential if stimulated with the appropriate growth factor combinations.

The long-term mast cell cultures were established with a high degree of efficiency from CD34$^+$ cord blood cells (5 of 5 cultures). Similar mast cell cultures were also established from CD34$^+$ cells purified from fetal blood or from purified murine adult stem cells (results not shown).

Previous studies showed that the in vitro growth of human mast cells required a murine stromal cell line for support.[17] Recently, Mitsui et al[18] showed that the growth factor produced by the murine stroma and primarily responsible for mast cell development is SCF. In fact, mononuclear cord blood cells growing in FBS with the addition of SCF would give rise by day 15 to differentiated cells which were shown functionally to be immature mast cells.[18] The mast cells proliferated for up to 2 months, after which bromodeoxyuridine incorporation was no longer detectable. Because the mast cells grown under these conditions remained immature in appearance and had a low proliferative index after several weeks in culture, it was speculated that SCF is primarily a maintenance factor.[18] In murine mast cells, IL-3 and SCF upregulate the expression of two different genes involved in the prevention of apoptosis: IL-3 upregulates the expression of bcl-2 and SCF upregulates the expression of p53.[38] These results would suggest that SCF and IL-3 are required at two different steps of the transduction pathway, which prevents apoptosis in murine mast cells.

In contrast with the work of Mitsui et al, we did not detect mast cells before 2 months of culture, and culture maintenance required both SCF and IL-3. One explanation of this difference could be that mast cell differentiation was triggered by two different progenitor cell populations in the

two studies. Light-density cells were used by Mitsui et al, and these cells contained progenitors that had the ability to rapidly differentiate and mature into mast cells. Our studies, which were performed with $CD34^+$ cells, may have been relatively enriched for primitive progenitor cells that required additional time in culture to express the mast cell phenotype.

We have now established long-term maintenance (>8 months) of human mast cells in serum-deprived cultures that was dependent on the presence of both SCF and IL-3. If either of these growth factors was removed from culture, the cultures could not be maintained. But even under these conditions, a high proportion of nonviable cells was found at each refeeding of the cultures, leading to a long (5- to 12-day) doubling time for the cultures and suggesting that additional growth factors are necessary for maintaining the viability of the cells or for permitting maturation in vitro. An IL-3–like factor and a factor capable of maintaining a higher proportion of mast cells might be produced by accessory cells or provided by the FBS in the culture system used by Mitsui et al. Two possible candidates for an autocrine mast cell growth factor are nerve growth factor (NGF) and IL-4. In fact, murine mast cells functionally express NGF and IL-4 receptors.[39-42] Furthermore, rat mast cells produce NGF in an autocrine fashion (R. Montalcini, personal communication) and human mast cells produce IL-4.[43] We are currently planning to evaluate the possibility of an autocrine loop involving IL-4 or NGF in the growth of human mast cells.

The results of our study have several implications. First, the fact that cord blood stem cells gave rise with high efficiency to mast cells that are not able to form colonies in semisolid medium suggests that immortalization, but not full transformation of these cells, had occurred in vitro. Furthermore, these cells are clearly growth factor dependent and, in fact, require multiple growth factors for maintenance. However, the fact that we were able to establish these long-term mast cell cultures with high efficiency raises a cautionary note about the potential long-term clinical use of SCF or strategies to expand stem cells in vitro using combinations of growth factors including SCF.

Second, the fact that many of the cells die under serum-deprived conditions, even in the presence of SCF and IL-3, suggests that other factors are necessary for the maturation and maintenance of viability of human mast cells. Thus, these cells may provide a tool to identify such growth factors.

Finally, it will be of interest to determine what the evolution of these human long-term cultures is after more time and whether changes in growth factor dependence (or the ability to grow in the absence of growth factors) is seen, suggesting further progression along the transformation pathway. To date, we have not seen such changes, but because these cells have an apparent normal karyotype and are not infected with the EBV, they could be a useful model for study of human tumorigenesis.

To our knowledge, these are the first human long-term mast cell cultures established and they should provide a useful tool for studies of mast cell differentiation as well as the biology of cord blood stem cells and progenitor cells that appear to be so easily maintained under these conditions.

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REFERENCES

1. Renauld JC, Goethals A, Houssiau F, Van Roost E, Van Snick J: Cloning and expression of a cDNA for the human homolog of mouse T cell and mast cell growth factor P40. Cytokine 2:9, 1990

2. Rennick DM, Lee FD, Yokota T, Arai KI, Cantor H, Nabel GJ: A cloned MCGF cDNA encodes a multilineage hematopoietic growth factor: Multiple activities of interleukin 3. J Immunol 134:910, 1985

3. Rennick D, Yang G, Muller-Sieburg C, Smith C, Arai N, Takabe Y, Gemmell L: Interleukin 4 (B-cell stimulatory factor 1) can enhance or antagonize the factor-dependent growth of hemopoietic progenitor cells. Proc Natl Acad Sci USA 84:6889, 1987

4. Thompson-Snipes L, Dhar V, Bond MW, Mosmann TR, Moore KW, Rennick DM: Interleukin 10: A novel stimulatory factor for mast cells and their progenitors. J Exp Med 173:507, 1991

5. Nocka K, Buck J, Levi E, Besmer P: Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. EMBO J 9:3287, 1990

6. Tsai M, Takeishi T, Thompson H, Langley KE, Zsebo KM, Metcalfe DD, Geissler EN, Galli SJ: Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. Proc Natl Acad Sci USA 88:6382, 1991

7. Tsai M, Shih LS, Newlands GFJ, Takeishi T, Langley KE, Zsebo KM, Miller HRP, Geissler EN, Galli SJ: The rat c-kit ligand, stem cell factor, induces the development of connective tissue-type and mucosal mast cells in vivo. Analysis by anatomical distribution, histochemistry, and protease phenotype. J Exp Med 174:125, 1991

8. Tsuji K, Zsebo KM, Ogawa M: Murine mast cell colony formation supported by IL-3, IL-4, and recombinant rat stem cell factor, ligand for c-*kit*. J Cell Physiol 148:362, 1991

9. Williams DE, Eisenman J, Baird A, Rauch C, Van Ness K, March CJ, Park LS, Martin U, Mochizuki DY, Boswell HS, Burgess GS, Cosman D, Lyman SD: Identification of a ligand for the c-kit proto-oncogene. Cell 63:167, 1990

10. Greenberger JS, Sakakeeny MA, Humphries RK, Eaves CJ, Eckner RJ: Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc Natl Acad Sci USA 80:2931, 1983

11. Palacios R, Karasuyama H, Rolink A: Lyl^+ PRO-B lymphocyte clones. Phenotype, growth requirements and differentiation in vitro and in vivo. EMBO J 6:3687, 1987

12. Spooncer E, Boettiger D, Dexter TM: Continuous in vitro generation of multipotential stem cell clones from src-infected cultures. Nature 310:228, 1984

13. Enver T, Nakamoto B, Karlinsey J, Josephson B, Greenberger J, Papayannopoulou T: Erythropoietin changes the globin program of an interleukin 3-dependent multipotential cell line. Proc Natl Acad Sci USA 85:9091, 1988

14. Migliaccio G, Migliaccio AR, Kreider BL, Rovera G, Adamson JW: Selection of lineage-restricted cell lines immortalized at different stages of hematopoietic differentiation from the murine cell line 32 D. J Cell Biol 109:833, 1989

15. Valtieri M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F,

Altmann S, Santoli D, Rovera G: Cytokine-dependent granulocytic differentiation: Regulation of proliferative and differentiative responses in murine progenitor cell line. J Immunol 138:3829, 1987

16. Hocking WG, Golde DW: Long-term human bone marrow cultures. Blood 56:118, 1980

17. Furitsu T, Saito H, Dvorak AM, Schwartz LB, Irani AM, Burdick JF, Ishizaka K, Ishizaka T: Development of human mast cells in vitro. Proc Natl Acad Sci USA 86:10039, 1989

18. Mitsui H, Furitsu T, Dvorak AM, Irani AMA, Schwartz LB, Inagaki N, Takei M, Ishizaka K, Zsebo KM, Gillis S, Ishizaka T: Development of human mast cells from umbilical cord blood cells by recombinant human and murine c-kit ligand. Proc Natl Acad Sci USA 90:735, 1993

19. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, Hsu RY, Birkett NC, Okino KH, Murdock DC, Jacobsen FW, Langley KE, Smith KA, Takeishi T, Cattanach BM, Galli SJ, Suggs SV: Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. Cell 63:213, 1990

20. Valent P, Spanblöchl E, Sperr WR, Sillaber C, Zsebo KM, Agis H, Strobl H, Geissler K, Bettelheim P, Lechner K: Induction of differentiation of human mast cells from bone marrow and peripheral blood mononuclear cells by recombinant human stem cell factor/*kit*-ligand in long-term culture. Blood 80:2237, 1992

21. Medlock ES, Mineo C, Housman JM, Elliott GS, Trebasky LD, Langley KE, Zsebo KM: Isolation of rat bone marrow mast lineage cells using Thy 1.1 and rat stem cell factor. J Cell Physiol 153:498, 1992

22. Durand B, Eddleman K, Migliaccio AR, Migliaccio G, Adamson JW: Long-term generation of colony-forming cells (CFC) from $CD34^+$ human umbilical cord blood cells. Leuk Lymphoma 11:263, 1993

23. Migliaccio G, Migliaccio AR, Druzin ML, Giardina PJV, Zsebo KM, Adamson JW: Long-term generation of colony-forming cells in liquid culture of $CD34^+$ cord blood cells in the presence of recombinant human stem cell factor. Blood 79:2620, 1992

24. Hows JM, Bradley GA, Marsh JCW, Luft T, Coutinho L, Testa NG, Dexter TM: Growth of human umbilical-cord blood in long-term haemopoietic cultures. Lancet 340:73, 1992

25. Migliaccio G, Migliaccio AR, Adamson JW: In vitro differentiation of human granulocyte/macrophage and erythroid progenitors: Comparative analysis of the influence of recombinant erythropoietin, G-CSF, GM-CSF, and IL-3 in serum-supplemented and serum-deprived cultures. Blood 72:248, 1988

26. Reisner Y, Kapoor N, Hodes MZ, O'Reilly RJ, Good RA: Enrichment for CFU-C from murine and human bone marrow using soybean agglutinin. Blood 59:360, 1982

27. Migliaccio G, Migliaccio AR, Druzin ML, Giardina PJV, Zsebo KM, Adamson JW: Effects of recombinant human stem cell factor (SCF) on the growth of human progenitor cells in vitro. J Cell Physiol 148:503, 1991

28. Migliaccio G, Migliaccio AR: Cloning of human erythroid progenitors (BFU-E) in the absence of fetal bovine serum. Br J Haematol 67:129, 1987

29. Chomcynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156, 1987

30. Arcari P, Martinelli R, Salvatore F: The complete sequence of a full length cDNA for human liver glyceraldehyde-3-phosphate dehydrogenase: Evidence for multiple mRNA species. Nucleic Acids Res 12:9179, 1984

31. Herrmann BG, Frischauf AM: Isolation of genomic DNA. Methods Enzymol 152:180, 1987

32. Zucker-Franklin D: Ultrastructural evidence for the common origin of human mast cells and basophils. Blood 56:534, 1980

33. Dombrowicz D, Flamand V, Brigman KK, Koller BH, Kinet JP: Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor a chain gene. Cell 75:969, 1993

34. Miyazawa K, Toyama K, Gotoh A, Hendrie PC, Mantel C, Broxmeyer HE: Ligand-dependent polyubiquitination of c-kit gene product: A possible mechanism of receptor down modulation in M07e cells. Blood 83:137, 1994

35. Yee NS, Langen H, Besmer P: Mechanism of kit ligand, phorbol ester and calcium-induced down-regulation of c-kit receptors in mast cells. J Biol Chem 268:14189, 1993

36. Yarden Y, Kuang WJ, Yang-Feng T, Coussens L, Munemitsu S, Dull TJ, Chen E, Schlessinger J, Francke U, Ullrich A: Human proto-oncogene *c-kit:* A new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J 6:3341, 1987

37. Carow CE, Hangoc G, Cooper SH, Williams DE, Broxmeyer HE: Mast cell growth factor (c-kit ligand) supports the growth of human multipotential progenitor cells with a high replating potential. Blood 78:2216, 1991

38. Yee NS, Paek I, Besmer P: Role of kit-ligand in proliferation and suppression of apoptosis in mast cells: Basis for radiosensitivity of W and Sl mutant mice. J Exp Med (in press)

39. Bischoff SC, Dahinden CA: Effect of nerve growth factor on the release of inflammatory mediators by mature human basophils. Blood 79:2662, 1992

40. Matsuda H, Kannan Y, Ushio H, Kiso Y, Kanemoto T, Suzuki H, Kitamura Y: Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. J Exp Med 174:7, 1991

41. Richard A, McColl SR, Pelletier G: Interleukin-4 and nerve growth factor can act as cofactors for interleukin-3–induced histamine production in human umbilical cord blood cells in serum-free culture. Br J Haematol 81:6, 1992

42. Horigome K, Pryor JC, Bullock ED, Johnson EM Jr: Mediator release from mast cells by nerve growth factor. Neurotrophin specificity and receptor mediation. J Biol Chem 268:14881, 1993

43. Brunner T, Heusser CH, Dahinden CA: Human peripheral blood basophils primed by interleukin 3 (IL-3) produce IL-4 in response to immunoglobulin E receptor stimulation. J Exp Med 177:605, 1993

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BRIEF NOTES

Steel Factor Is Required for Maintenance, but Not Differentiation, of Melanocyte Precursors in the Neural Crest

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Skin melanocytes are derived from neural crest cells that migrate into the dermis during embryogenesis. Two mouse mutants, *Steel* and *White dominant-spotting*, which have defects in melanocyte production, have recently been shown to have deletions in the genes that code for a new growth factor, steel factor (SLF), and its receptor, respectively. Here, we have investigated the role that SLF plays in melanogenesis using cultures of mouse neural crest and found that its primary action is the maintenance of melanocyte precursors. It has no effect on the final stage of melanocyte differentiation, the production of melanin, which appears to require an additional factor whose action is mimicked by the phorbol ester TPA (12-*O*-tetradecanoyl-phorbol-13-acetate). © 1992 Academic Press, Inc.

INTRODUCTION

A diverse array of cell types in the vertebrate are derived from the embryonic neural crest, including the peripheral nervous system, the adrenal medulla, the facial mesenchyme, and the melanocytes of the skin (Le Douarin, 1986; Weston, 1986). Melanocytes are apparently determined early in development (Mintz, 1967) and their precursors migrate laterally to the skin where they continue to proliferate and ultimately differentiate into pigmented melanocytes (Rawles, 1944; Weston, 1963).

The processes that control the proliferation, migration, and differentiation of melanocyte precursors are not clearly understood; however, two classes of mouse mutants have made it clear that an essential part of this process is mediated by a receptor–ligand interaction. Mice homozygous at either the *White dominant-spotting* (*W*) or *Steel* (*Sl*) gene locus are black-eyed white, anemic, and sterile, and sometimes die during embryogenesis (reviewed in Silvers, 1979; Russel, 1979; Geissler *et al.*, 1981). An analysis of these mutations has revealed a complementary molecular relationship between the two alleles: the *W* allele codes for a growth factor receptor-like tyrosine kinase, identical to the proto-oncogene *c-kit,* and the *Sl* locus encodes the ligand for *c-kit* also called steel factor (SLF), mast cell growth factor, and stem cell factor, reflecting its various biological activities (see Witte, 1990).

Although these *in vivo* studies establish the importance of SLF in melanogenesis, its precise role in this process remains unclear. In this paper we show that SLF in combination with the phorbol ester TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) stimulates the production of melanocytes in cultures of mouse neural crest. SLF appears to act primarily to maintain melanocyte precursor levels and does not promote the differentiation of the precursor into mature melanocytes, which is dependent on another factor presumably acting through the protein kinase C pathway.

MATERIALS AND METHODS

Neural crest cultures. Neural tubes were prepared from the thoracic and lumbar segments of Embryonic Day 9 (E9) CBA mice as described previously (Murphy *et al.*, 1991). One neural tube was placed in each well of a 24-well plate (Falcon) precoated with fibronectin (50 μg/ml) and cultured in Monomed medium (Commonwealth Serum Laboratories, Melbourne, Aus) containing 10% fetal bovine serum and growth factors as specified, in a humidified incubator at 37°C and 5% CO_2 in air.

Factors. TPA was obtained from Sigma (St. Louis, MO) and SLF (recombinant murine mast cell growth factor) from Immunex. Both factors were usually added only once to the cultures at the indicated times, except where otherwise indicated.

Histochemistry. Cultures were fixed with 10% formalin 2 weeks after plating and the total number of melanin-containing melanocytes was determined for each culture under bright-field microscopy. Subsequently,

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after careful washing with phosphate-buffered saline (PBS), some of the cultures were treated with 0.1% 2-β-(3,4)-dihydroxyphenylalanine (DOPA, BDH, Poole, England) in PBS for 2 hr at room temperature to reveal latent melanocytes (Ito and Takeuchi, 1984). The reaction was then stopped by washing the cultures three times with PBS. The cultures were then recounted to ascertain the total melanocyte number. Some cultures were subsequently stained with 0.1% cresyl violet in water to reveal neurons.

RESULTS AND DISCUSSION

Although large numbers of cells migrated and developed in neural crest cultures after 2 weeks when grown in medium containing 10% serum, none of the cultures contained pigmented cells. This was in spite of the fact that the same cultures gave rise to neurons and Schwann cells during the observation period. Thus, this culture system can support the growth of neural crest cells and, to a certain extent, their differentiation into some neural crest derivatives, but with no endogenous potential for melanogenic differentiation. Others find some melanocyte differentiation in mouse neural crest cultures in the presence of embryo extract (Ito and Takeuchi, 1984; Morrison-Graham *et al.*, 1990), which we have not used in our cultures.

We first tested whether SLF could stimulate the production of melanocytes in these cultures by adding it at the time of plating at a concentration of 100 ng/ml, a concentration found to provide maximum stimulation of the mast cell line, MC-6 (Williams *et al.*, 1990). However, SLF did not induce melanocyte production, with or without DOPA enhancement, and had no observable effect on the growth or appearance of the cultures (data not shown and Fig. 2). Thus, SLF alone was not sufficient to stimulate melanocyte differentiation.

Previously, the phorbol ester TPA was shown to stimulate human melanocyte growth (Eisenger and Marko, 1982; Halaban *et al.*, 1983) and also to stimulate melanocyte production in cultures of avian dorsal root ganglia (Ciment *et al.*, 1986). Therefore, we investigated the effects of TPA on the development of melanocytes in mouse neural crest cultures. The addition of TPA (10–100 nm) to neural crest cultures at Day 1 gave rise to both pigmented and DOPA-positive melanocytes that began to appear after 7–8 days of culture. These melanocytes were not evenly distributed through the cultures but were found in discrete areas and always associated with a sheet of neuroepithelial cells that grew out from the neural tube (Figs. 1a, c, and e show clusters of melanocytes). They were never found beyond the perimeter of the epithelial sheet or at its edge. There was also considerable variability in the total number of melanocytes that arose in each culture (Fig. 2A and see below). This association of melanocytes with neuroepithelial sheet and variability in number has been observed in previous studies of melanocyte differentiation in the mouse (Ito and Takeuchi, 1984; Morrison-Graham *et al.*, 1990). In cultures where little or no epithelial sheet had grown out from the explants there were few if any melanocytes. The importance of neuroepithelium was confirmed by the finding that cultures in which the neural tube was removed after 24 hr (leaving only migrated neural crest cells) failed to produce melanocytes, even under conditions that gave maximum melanocyte numbers as described below (data not shown).

Since conditions permitting melanocyte differentiation in neural crest cultures had been established, the effect of SLF was reexamined. SLF and TPA were added simultaneously to the neural crest cultures at Day 1 of culture and after approximately 1 week melanocytes appeared in the cultures and continued to increase in number over the following week. However, the distribution of melanocytes in these cultures was quite different from that seen in cultures treated with TPA alone. In the SLF + TPA cultures melanocytes were present in association with the neuroepithelial sheet, but in addition they extended out into the area where the neural crest cells had migrated beyond the epithelium and were particularly dense at the interface between the epithelial sheet and the neural crest cells (Figs. 1b, d, and f). In cultures treated only with TPA, melanocytes were found only in association with the neuroepithelial sheet, as previously discussed (Figs. 1a, c, and e). Whether this different distribution of melanocytes is due to an increase in a subpopulation of melanocytes arising in the SLF + TPA cultures or to other reasons, such as an increase in the migration of the melanocytes away from the neural tube, is currently being investigated.

The total number of melanocytes in the SLF + TPA-treated cultures also appeared to be greater than in the cultures treated with TPA alone. In order to assess this difference, the total number of melanocytes were counted in each neural crest culture. In these cultures, melanocytes were counted only where a clear cell body with at least one dendritic process could be observed by microscopy. In some of the cultures treated with SLF + TPA, dark clumps of melanocytes arose or were densely intermingled (Figs. 1b, d, and f) and could not be counted. In five different experiments (each of five or six replicates) the number of melanocytes was counted and the pooled data showed an overall 9.2-fold increase ($P < 0.001$, t test) in the number of melanocytes in SLF + TPA-treated cultures (mean = 313 ± 247 melanocytes, n = 27) compared to TPA-treated cultures (mean = 34 ± 60 melanocytes, n = 28). This may be an underestimate of

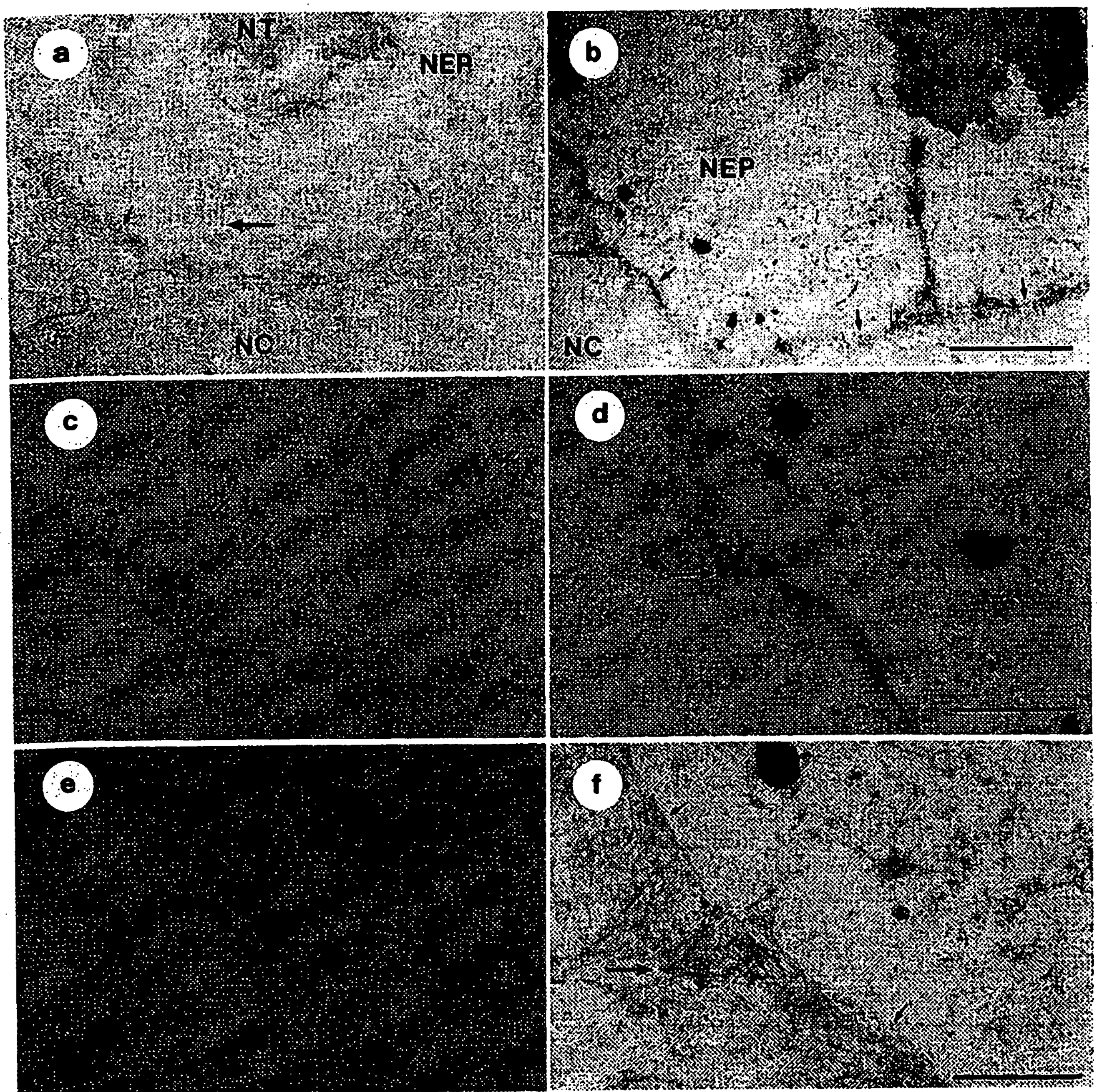

FIG. 1. Melanocyte production in 2-week-old neural crest cultures treated with 10 n*M* TPA (a, c, e) or TPA plus 100 ng/ml SLF (b, d, f). Cultures were fixed and photographed with no DOPA treatment at low, medium, and high power. The large arrows indicate particular areas of melanocyte distribution. As can be seen at lower magnifications in the TPA-alone cultures (a, c), the melanocyte distribution is restricted to regions of neuroepithelium (NEP, boundary of neuroepithelial sheet demarcated by small arrows), whereas in the TPA + SLF-treated cultures many melanocytes are also found either at the interface between neuroepithelial sheet and neural crest cells or beyond the epithelial boundary in regions composed mainly of neural crest cells (NC; b, d, f). NT, neural tube. Bars = 0.7 mm (a, b); 0.28 mm (c, d); and 0.14 mm (e, f).

the difference in melanocyte numbers between the two conditions, because of the difficulty in accurately determining the number of melanocytes contained in densely aggregated regions in the SLF + TPA cultures.

Some of the cultures were treated with DOPA to reveal latent melanocytes. After DOPA treatment, more melanocytes could be seen in all of the cultures (compare Fig. 2a to Fig. 2b). However, the ratio of melanocyte number in TPA cultures to that in SLF + TPA cultures was similar in magnitude to the ratio of melanocyte number in cultures before DOPA treatment to that after DOPA treatment (Fig. 2b), and the DOPA treatment did not reveal any other differences in the cultures.

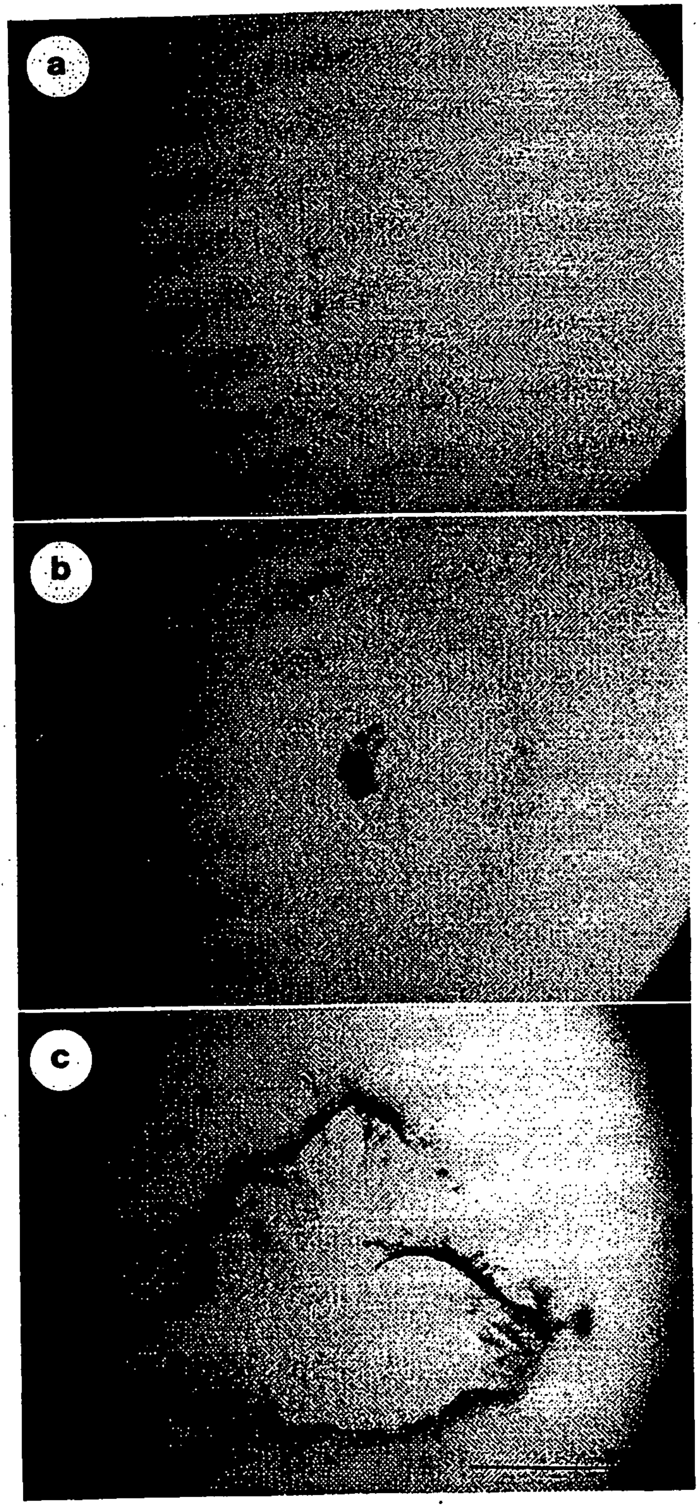

FIG. 4. Photomicrographs of entire neural crest cultures treated with TPA alone or with SLF + TPA. Neural crest cells were cultured in the presence of TPA alone (a), TPA and one dose of SLF (b), or TPA with refeeding of SLF every 2-3 days (c) for 2 weeks, fixed, and photographed. Individual melanocytes cannot be visualized at this magnification; only groups of melanocytes are visualized. Bar = 2 mm.

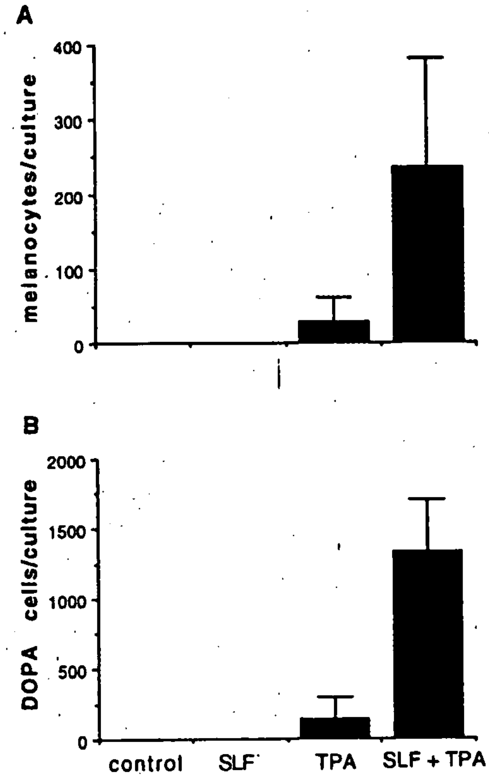

FIG. 2. Effect of SLF and TPA on the total number of melanocytes generated per individual neural crest culture. Cultures were treated as in Fig. 1, but melanocytes were counted before (A) and after treatment with DOPA (B). Values are the means and standard deviation obtained from six replicates.

We next explored the idea that the observed synergy between TPA and SLF in the production of melanocytes resulted not from an increase in the magnitude of the differentiation signal given to the precursor, but from each factor's supporting a different phase of melanogenesis. This was done by first adding SLF to the cultures at the time of plating and, after either 11 or 14 days, removing the SLF by washing and then adding TPA. This regimen was chosen as it provided a system free of background melanogenesis. That is, SLF alone had no effect (as shown in Fig. 2), and melanocytes arose only rarely in SLF-free cultures in which the addition of TPA was delayed beyond 11 days of culture (Fig. 3), most likely because the melanocyte precursors had died by this time. Under these conditions, melanocytes appeared as early as 1 day after addition of TPA and increased in number over the week before fixation and scoring (Fig. 3). Cultures from which the SLF was not washed out after 11 days gave similar results (Fig. 3). These results confirmed that SLF does not have a direct effect on melanocyte differentiation and suggest that it acts primarily to maintain the melanocyte precursors *in vitro*.

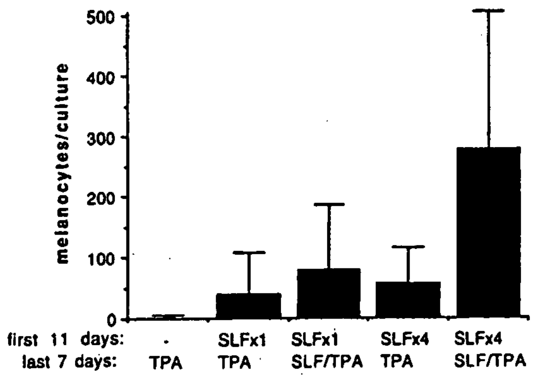

FIG. 3. Effect of SLF on the survival of melanocyte precursors. Melanocyte numbers were determined without DOPA treatment 18 days after the commencement of culture and 7 days after the addition of TPA. Cultures either contained no SLF (−) or received SLF on Day 1 only (SLF × 1) or every 2–3 days (SLF × 4) for the first 11 days. In addition, some cultures received additional SLF during the final 7 days of TPA treatment (SLF/TPA). The numbers of melanocytes in at least 12 and up to 19 cultures were determined for each condition. All conditions in which cultures were treated with SLF had significantly more melanocytes than those in which cultures were treated with TPA alone ($P < 0.0001$, determined according to a Mann-Whitney *U*/Wilcoxon rank sum *W* test). It was also found that cultures treated with SLF for the entire 18-day period (SLF × 4, SLF/TPA) had significantly higher levels of melanocytes than any other condition ($P < 0.004$).

In these pulsing experiments, not as many melanocytes appeared as in those experiments in which SLF and TPA were present from the start. Therefore, it was possible that SLF added to the cultures at Day 1 was not sufficient to sustain melanocyte precursors for the whole culture period. We tested this possibility by repeating the above experiments but adding fresh SLF to the cultures every 2–3 days for the entire 18 days of culture and found a significant increase in melanocytes (Fig. 3, mean = 278), which was similar to the number observed when SLF and TPA were present from the start (Fig. 2A, mean = 313). Cultures that were fed SLF continuously for the first 11 days of culture then had it removed upon addition of TPA contained significantly fewer melanocytes than those fed SLF for the entire 18-day period (Fig. 3). This further suggests that melanocyte precursors require SLF for survival before they have differentiated and that TPA acts as a differentiation agent and is not sufficient for precursor survival.

These results prompted us to culture the neural crest cells in the presence of TPA and refeed with SLF every 2–3 days; these conditions produced the greatest number of melanocytes as assessed by visual comparison between the cultures. In these cultures the areas containing melanocytes were clearly visible macroscopically compared to cultures treated with TPA alone or with with one dose of SLF + TPA (and see Fig. 4 for a view of the entire neural crest cultures), but the melanocytes were too densely intermingled to accurately quantitate.

As there are no specific markers for melanocyte precursors it is difficult to assess whether SLF action is restricted to promoting survival or also promotes precursor proliferation. A number of observations suggest that SLF action is likely to be predominantly one of maintenance. First, the majority of neural crest cells divide in SLF-free cultures [with the exception of sensory neuron precursors (Murphy *et al.*, 1991)], so it seems likely that melanocyte precursors proliferate without SLF. Second, since no increase in the number of melanocytes occurred in cultures continuously treated with SLF in the absence of TPA (where the precursors were not differentiating) compared to those treated with SLF in the presence of TPA (where the precursors were differentiating), it appeared that no expansion of the precursor population had occurred as would be predicted if SLF were a precursor mitogen. Cultures that were constantly exposed to both SLF and TPA probably gave rise to most melanocytes (Fig. 4), possibly because TPA acts not only to stimulate melanocyte differentiation but also to stimulate proliferation of melanocytes once they have differentiated, as previously shown for human melanocytes (Eisenger and Marko, 1982; Halaban *et al.*, 1983).

There are alternative explanations for the action of SLF in these neural crest cultures. For example, SLF may function to stimulate pluripotent stem cell proliferation, such that the addition of a lineage-specific differentiation factor would increase that subpopulation. However, there are two arguments against this possibility. First, despite careful observation, we could only detect differences in melanocyte number and not in other crest cell derivatives such as neurons (detected after Nissl staining) and Schwann-like cells (as detected by characteristic morphology and after staining with an S-100 antibody; data not shown). Second, the only neural crest cell derivative known to be affected in *Sl* and *W*/*W* mice is the melanocyte and if SLF did function on such pluripotent stem cells, the mutations in these mice would be expected to have much more widespread effects.

The notion that SLF acts as a maintenance factor for melanocyte precursors fits well with its known distribution *in vivo*. High levels of SLF expression have been found in the dorsal regions of the somites over which the melanocyte precursors migrate (much lower levels are found in other regions of the somite through which other lineages of neural crest cells migrate); high levels are also found in areas to which melanocyte precursors migrate, for example, dermis of the skin and whisker follicles (Matsui *et al.*, 1990). In addition, recent experiments using a monoclonal antibody to the SLF receptor,

c-kit, *in vivo* show a critical period for the action of SLF around E14.5 (Nishikawa *et al.*, 1991), which is before melanocyte differentiation occurs, and is thus consistent with the results we find here. SLF is also expressed in the floor plate of the neural tube at the time of neural crest migration (Matsui *et al.*, 1990). This neuroepithelial source of SLF may explain why exogenous SLF is not an absolute requirement in the early stages of our cultures where the precursors remain close to the tube.

The *in vitro* results also infer that there is second signal that is mimicked by TPA which is produced at the site of melanocyte differentiation. As TPA activates the protein kinase C pathway, any number of growth factors, hormones, or membrane interactions may be responsible for this final stimulus. One possible candidate, melanocyte-stimulating hormone (see Ito and Takeuchi, 1984), does not appear to influence melanogenesis in our cultures (data not shown) and so the identity of this factor remains unknown. It is possible that the second factor is present in small levels in mouse and chick embryo extracts as discussed above (Ito and Takeuchi, 1984; Morrison-Graham *et al.*, 1990).

While these experiments strongly suggest a role for SLF at the melanocyte precursor stage, there are presumably a number of stages of development that the melanocyte precursor goes through from uncommitted neural crest precursor cell to the stage just before final melanocyte differentiation. The next task will be to define the exact stage at which SLF functions. Experiments such as single-cell cloning or culture of *Steel* mutant neural crest may help to define this stage.

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REFERENCES

Ciment, G., Glimelius, B., Nelson, D. M., and Weston, J. A. (1986). Reversal of a developmental restriction of neural crest-derived cells of avian embryos by a phorbol ester drug. *Dev. Biol.* 118, 392–398.

Eisenger, M., and Marko, O. (1982). Selective proliferation of normal human melanocytes *in vitro* in the presence of phorbol ester and cholera toxin. *Proc. Natl. Acad. Sci. USA* 79, 2018-2022.

Geissler, E. N., McFarland, E. C., and Russell, E. S. (1981). Analysis of the pleiotropism at the dominant *white-spotting* (*W*) locus of the house mouse: A description of ten new *W* alleles. *Genetics* 97, 337–361.

Halaban, R., Pomerantz, S. H., Marshall, S., Lambert, D. T., and Lerner, A. B. (1983). The regulation of tyrosinase in human melanocytes grown in culture. *J. Cell Biol.* 97, 480–488.

Ito, K., and Takeuchi, T. (1984). The differentiation *in vitro* of the neural crest cells of the mouse embryo. *J. Embryol. Exp. Morphol.* 84, 49–62.

Le Douarin, N. M. (1986). Cell line segration during peripheral nervous system ontogeny. *Science* 231, 1516–1522.

Matsui, Y., Zsebo, K. M., and Hogan, B. M. (1990). Embryonic expression of a haematopoietic growth factor encoded by the *Sl* locus and the ligand for *c-kit*. *Nature* 347, 667–669.

Mintz, B. (1967). Gene control of mammalian pigmentary differentiation. 1. Clonal origin of melanocytes. *Proc. Natl. Acad. Sci. USA* 58, 344-351.

Morrison-Graham, K., West-Johnsrud, and Weston, J. A. (1990). Extracellular matrix from normal but not mutant mice enhances melanogenesis in cultured mouse neural crest cells. *Dev. Biol.* 139, 299-307.

Murphy, M., Reid, K., Hilton, D. J., and Bartlett, P. F. (1991). Generation of sensory neurons is stimulated by leukemia inhibitory factor. *Proc. Natl. Acad. Sci. USA* 88, 3498–3501.

Nishikawa, S., Kusajabe, M., Yoshinaga, K., Ogawa, M., Hayasha, S-I., Kunisada, T., Era, T., Sakakura, T., and Nishikawa, S-I. (1991). *In utero* manipulation of coat color formation by a monoclonal anti-*c-kit* antibody: Two distinct waves of *c-kit*-dependency during melanocyte development. *EMBO J.* 10, 2111–2118.

Rawles, M. E. (1944). The migration of melanoblasts after hatching into pigment-free skin grafts of the common fowl. *Physiol. Zool.* 17, 167–183.

Russel, E. S. (1979). Hereditary anemias of the mouse: A review for geneticists. *Adv. Genet.* 20, 357–459.

Silvers, W. K. (1979). White-spotting, patch and rump-white; steel, flexed tail, splotch and variant-waddler. *In* "The Coat Colors of Mice: A Model for Gene Action and Interaction," pp. 206–241. Springer-Verlag, New York.

Weston, J. A. (1963). A radioautographic analysis of the migration and localization of trunk neural crest cells in the chick. *Dev. Biol.* 6, 279–310.

Weston, J. A. (1986). Phenotypic diversification in neural crest derived cells: The time and stability of commitment during early development. *Curr. Top. Dev. Biol.* 20, 195–210.

Williams, D. E., Eisenman, J., Baird, A., Rauch, C., Van Ness, K. V., March, C. J., Park, L. S., Martin, U., Mochizuki, D. Y., Boswell, H. S., Burgess, G. S., Cosman, D., and Lyman, S. D. (1990). Identification of a ligand for the *c-kit* proto-oncogene. *Cell* 63, 167–174.

Witte, O. N. (1990). Steel locus defines new multipotent growth factor. *Cell* 63, 5–6.

3. Geissler, E., Ryan, M. & Houseman, D. *Cell* **55**, 185–192 (1988).
4. Orr-Urtreger, A. *et al. Development* **109**, 911–923 (1990).
5. Zsebo, K. M. *et al Cell* **63**, 195–201 (1990).
6. Martin, F. H. *et al. Cell* **63**, 203–211 (1990).
7. Zsebo, K. M. *et al. Cell* **63**, 213–224 (1990).
8. Matsui, Y., Zsebo, K. M. & Hogan, B. L. M. *Nature* **347**, 667–669 (1990).
9. Godin, I., Wylie, C. C. & Heasman, J. *Development* **108**, 357–363 (1990).
10. Nocka, K., Buck, J., Levi, E. & Besmer, P. *EMBO J* **9**, 3287–99 (1990).
11. Anderson, D. *et al. Cell* **63**, 235–243 (1990).
12. Dolci, S. *et al. Nature* **352**, 809–811 (1991).
13. Dexter, T. M., Heyworth, C. M., Spooncer, E. & Ponting, I. L. *Phil. Trans. R. Soc.* B **327**, 85–98 (1990).
14. Donovan, P., Stott, D., Cairns, L. A., Heasman, J. & Wylie, C. C. *Cell* **44**, 831–838 (1986).
15. Maniatis, T., Fritsch, E. & Sambrook, J. *Molecular Cloning* (Cold Spring Harbor Laboratory, New York, 1987).

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Requirement for mast cell growth factor for primordial germ cell survival in culture

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MAST-CELL growth factor (MGF) is encoded by the murine *steel* (*Sl*) locus and is a ligand for the tyrosine kinase receptor protein encoded by the proto-oncogene c-*kit* at the murine *dominant white spotting* (*W*) locus. Mutations at both these loci affect mast cells, primordial germ cells (PGCs), haemopoietic stem cells and melanocytes. In many *Sl* and *W* mutants, the rapid proliferation of PGC that normally occurs between day 7 and 13.5 of embryonic development fails to occur. As c-*kit* is expressed in PGCs[1,2] while MGF is expressed in the surrounding mesenchyme[2,3], MGF might promote the proliferation of PGCs. Here we report that MGF is essential for PGC survival in culture, but does not stimulate PGC proliferation. Moreover, whereas both the transmembrane and soluble proteolytic cleavage forms of MGF stimulate mast-cell proliferation, soluble MGF has a relatively limited ability to support survival of PGCs in culture, thus explaining the sterility in mice carrying the *steel-dickie* (Sl^d) mutation, which encodes only a soluble form of MGF, and providing a functional role for a transmembrane growth factor.

The ability of MGF to promote survival and/or proliferation of PGCs was determined using a culture system in which both the type and amount of MGF present could be manipulated. Isolated pregonadal (8.5–10.5 days) PGCs survive <24 h in culture in the absence of a confluent monolayer of feeder cells[4]. PGCs do survive when cultured on feeder layers of STO or NIH-3T3 cells[4-6] but not on feeder layers of CV-1 cells (Fig. 1*a*). The differences in PGC survival on STO and NIH-3T3 versus CV-1 feeder layers might be due to differential MGF expression by these feeder cell lines. STO and NIH-3T3 cells both express a 6.5-kilobase (kb) MGF messenger RNA and produce MGF that promotes the proliferation of mast cells, whereas CV-1 cells do neither (Fig. 1*b*; Table 1). CV-1 cells

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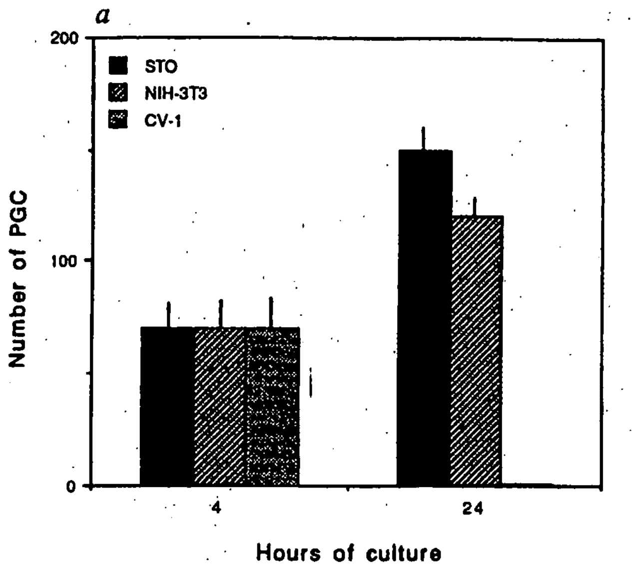

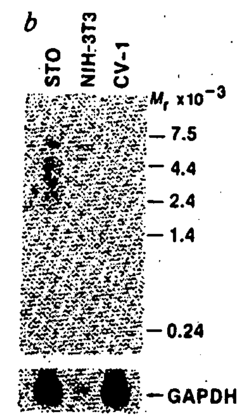

FIG. 1 *a*, Survival of PGCs on STO, NIH-3T3 or CV-1 feeder layers. The number of PGCs present after 4 and 24 h of culture. Bars represent the mean plus/minus the standard deviation of five replicate cultures. Each experiment was done four times. Solid bar, STO; hatched bar, NIH-3T3; dotted bar, CV-1. *b*, Northern analysis of MGF expression by feeder cell lines, STO cells, NIH-3T3 cells and CV-1 cells. RNA loading was assayed by probing for glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

METHODS. STO cells and NIH-3T3 cells are mouse embryo-derived fibroblast cell lines. CV-1 cells are fibroblast-like cells derived from the kidney of a male African green monkey. Cells were grown in DMEM (Gibco) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and either 10% FBS (STO and CV-1) or 10% calf serum (NIH-3T3 cells). Staged embryos were derived from timed matings of B6C3F1 animals as described[4], and PGCs isolated from 8.5-day postcoitus (dpc) embryos by dissection of a fragment consisting of the caudal end of the primitive streak and the base of the allantoic rudiment[6]. These fragments were dissociated in 0.05% trypsin, 0.02% EDTA (Sigma) to yield a single-cell suspension of PGC and somatic cells. This cell suspension was plated onto confluent, irradiated, feeder layers as previously described[4]. PGC were maintained in feeder-layer culture in DMEM supplemented with 15% FBS and identified by alkaline phosphatase histochemistry[4,6]. For northern analyses, confluent cell monolayers were washed with PBS, lysed with RNAzol (Cinna/Biotex) and total RNA prepared by the method of Chomczynski and Sacchi[22]. Poly(A)$^+$ RNA was selected using a Fast Track kit (InVitrogen). Poly(A)$^+$ RNA (2 μg) were run on a 1.5% agarose-formaldehyde gel, transferred to Nytran (Schleicher and Schuell) and baked. Northern blots were probed with a 2.0-kb *Sal*I fragment of an MGF cDNA clone (MGF-10), representing the entire MGF coding sequence[15]. The probe was ^{32}P-labelled using a Multiprime labelling kit (Amersham) and hybridizations were done according to Church and Gilbert[23]. To quantitate RNA loading, blots were stripped and reprobed with a GAPDH cDNA probe[24].

transfected with a construct encoding transmembrane MGF (CV-1-Wt) are able to stimulate mast-cell proliferation and support PGC survival for at least 3 days (Table 1; Fig. 2*a*). Taken together these data strongly suggest that MGF mediates PGC survival in culture.

Although MGF is required for survival it is not sufficient for proliferation of PGCs in culture. PGCs survive and proliferate on feeder layers of STO cells (Fig. 2*b* and refs 4-6). On NIH-3T3 cells, PGCs survive for up to 5 days, but their numbers gradually decline and their proliferation rate, assessed by 5-bromo-2'-deoxyuridine (BrdU) incorporation, is about 50% that of PGCs cultured on STO cells (Fig. 2*b*; data not shown). Addition of a recombinant form of MGF (rMGF) lacking the transmembrane domain and cytoplasmic tail has no effect on PGC proliferation on NIH-3T3 cells, despite its abilty to stimulate mast-cell proliferation (Fig. 2*b*). Similarly, Godin *et al.*[7] conclude that added soluble factor does not stimulate proliferation of PGCs cultured on STO cells. Apparently, PGC proliferation on STO cells is not simply due to higher levels of soluble MGF. STO cells either produce higher levels of transmembrane MGF, a unique form of MGF or, in addition to MGF, another factor which is able to stimulate PGC proliferation. Addition of STO cell-conditioned medium to NIH-3T3 cells stimulated PGC proliferation suggesting that STO cells produce, in addition to MGF, a soluble PGC mitogen (data not shown).

Many lethal *Sl* mutants are completely deleted for MGF coding sequences[8-10]. By contrast, the viable, but sterile, Sl^d mutant is deleted only for sequences encoding the MGF cytoplasmic tail and transmembrane domain[11,12]. In Sl/Sl^d mice, PGC form and migrate, but the few PGC that colonize the gonad anlagen do not survive[13]. As Sl^d can produce only a soluble form of MGF; soluble MGF may be unable to support long-term survival of PGC. To test this idea, we compared PGC survival on CV-1 cells transfected with a construct encoding a truncated MGF identical to that encoded by the Sl^d allele (CV-1-Sl^d) and CV-1 cells transfected with the transmembrane MGF (CV-1-Wt). As CV-1 cells seem unable to cleave transmembrane MGF to the soluble form (D.E.W., unpublished observations) CV-1-Wt probably produce only transmembrane MGF whereas CV-1-Sl^d cells produce only soluble MGF. CV-1-Sl^d supported PGC survival to 70% of CV-1-Wt after 1 day ($P<0.005$) and 40% of CV-1-Wt after 3 days ($P<0.038$; Fig. 2*a*), despite the fact that

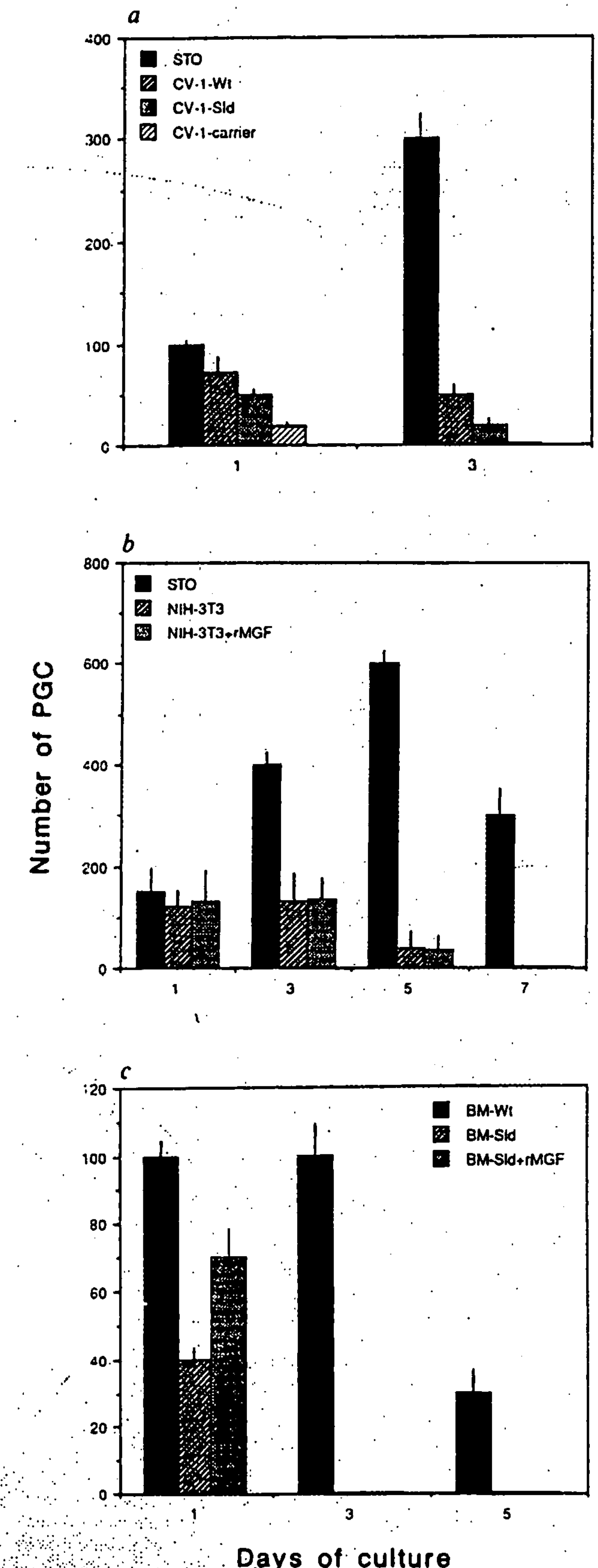

FIG. 2 Analysis of the survival and proliferation of PGCs in culture. *a*, The number of PGCs present on days 1 and 3 of culture on STO cells and CV-1 cells transfected with constructs encoding wild-type MGF (CV-1-Wt), Sl^d MGF (CV-1-Sl^d), or carrier DNA. This experiment was repeated four times. MGF expression was assayed as previously described[14] using a mast cell line, MC-6, which is responsive to MGF (see Table 1). *b*, The number of PGC present at days 1, 3, 5 and 7 of culture on STO cells, NIH-3T3 cells and NIH-3T3 cells to which 100 ng ml^{-1} rMGF was added. This experiment was repeated three times. *c*, The number of PGC present after 1, 3 and 5 days of culture on bone marrow stromal cell lines derived from wild-type (BM-Wt) and Sl/Sl^d (BM-Sl^d) mice, and BM-Sl^d cells to which 100 ng ml^{-1} rMGF was added. Bars represent the mean ± the standard deviation of five replicate cultures. This experiment was repeated four times.

METHODS. Brains were taken from wild-type (C57BL/6J) or Sl/Sl^d (DBA) animals. Total RNA was prepared as above using RNAzol (Cinna/Biotecx)[22]. Each RNA was used as a template to synthesize single-stranded cDNA with the Copy Kit (InVitrogen). These cDNAs were used as a template in a polymerase chain reaction (PCR) with the amplimers: 5'-CGGAGTCGCC-ACACCGCTGC-3' and 5'-GTCACTTGAGACAGAATGGG-3' (400 ng each) with the following conditions: 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. Each reaction product was gel-purified, kinased, filled in with T4 polymerase and ligated into Bluescript (Stratagene). Each clone was sequenced to verify authenticity; Wt, 956 base pairs (bp) and Sl^d, 782 bp. MGF was expressed from the SV40 early promoter vector pSG5 (Stratagene). An *XhoI* linker was placed in the pSG5 *Bam*HI site, and the *XhoI–Bam*HI polylinker sites of pBluescript SK (Stratagene) transferred into the pSG5 *XhoI* and *BglII* sites. *XhoI–Bam*HI fragments from PCR reactions of the Wt and Sl^d alleles were then cloned into these expanded polylinkers. CV-1 cells on 10-cm dishes were transfected with 20 μg expression vector by the calcium phosphate technique[25]. The cells were shocked with 15% glycerol 18 h after the addition of precipitate. Bone marrow stromal cells were cultured in McCoy's 5A medium (Gibco) supplemented with 10% heat-inactivated FBS as previously described[14,26]. PGCs were isolated as previously described (see above) and plated onto confluent monolayers of stromal cells that had been irradiated with 5,000 rads to induce quiescence. Cultures were maintained in DMEM plus 15% FBS (as described earlier). The rMGF was produced as previously described[15] and used over a concentration range of 0.001–1 μg ml^{-1}.

TABLE 1 Proliferation of the MGF-dependent mast cell line MC-6 on established cell lines and transfected CV-1 cells

Cell line	^{3}H-thymidine incorporation (c.p.m.)
STO	6,728 ± 2,413*
NIH-3T3	9,849 ± 3,839†
CV-1	2,064 ± 138
CV-1 WT MGF	7,552 ± 575†
CV-1 Sl^d MGF	13,244 ± 1,917†
CV-1 + carrier	2,365 ± 492

Proliferation assays were essentially carried out as previously described[14]. Briefly, 20,000 feeder cells were plated into microtitre wells and irradiated with 5,000 rads to induce quiesence. After 24 h, 10,000 MC-6 cells were added to each well and after a further 24 h the cultures were pulsed for 4-5 h with 1 μCi [^{3}H]thymidine. Wells were collected on fibreglass filters with an automated collector and incorporated [^{3}H]thymidine counted by liquid scintillation spectrometry. This experiment was repeated four times with six replicates per treatment.

* $P \leq 0.05$ versus CV-1 cells or CV-1 + carrier.

† $P \leq 0.001$ versus CV-1 cells or CV-1 + carrier.

the total MGF produced by CV-1-Sl^d (assayed by mast cell proliferation) was higher than that of CV-1-Wt (Table 1). We also compared PGC survival on Sl/Sl^d (BM-Sl^d) and congenic wild-type (BM-Wt) bone marrow-derived stromal cell lines. BM-Sl^d supported PGC survival to 40% of BM-Wt after 1 day, but did not support subsequent PGC survival (Fig. 2*c*). When recombinant MGF (rMGF) was added to PGCs cultured on BM-Sl^d, PGC survival of over one day improved to 70% of BM-Wt, but subsequent PGC survival was unaffected (Fig. 2*c*). When rMGF was added to PGCs cultured on NIH-3T3 or STO cells, PGC survival was not enhanced (Fig. 2*b* and data not shown). Presumably, in the culture conditions used in this study, the amount of MGF produced by NIH-3T3 and STO cells is sufficiently high to support maximal PGC survival in the absence of added rMGF (see Fig. 1*b* and Table 1). Soluble MGF, such as that produced by Sl/Sl^d cells, is able to support only limited PGC survival and is unable to support long-term survival seen when PGC are cultured on wild-type cells producing both transmembrane and soluble MGF. In a similar study, Godin *et al.*[7] report that soluble factor is able to enhance initial survival (>48 h) but does not promote long-term survival of PGC cultured in the absence of STO cells.

The reduced ability of soluble MGF to support long-term survival may reflect a requirement for a localized, high concentration of MGF, for a particular conformation of MGF, for a role of MGF in promoting cell adhesion, or for an extended ligand-receptor interaction precluded by internalizable, soluble MGF. That transmembrane MGF is more effective in supporting PGC survival could provide (1) a mechanism both for the haptotactic guidance of PGCs to the gonad anlagen as well as for strict regulation of PGC proliferation and differentiation in the embryo and (2) a possible explanation for the sterility found in Sl/Sl^d mice. The viability, but sterility and lack of pigment, of Sl/Sl^d mice suggests that the haemopoietic lineages can be maintained to some extent by soluble factor, whereas PGCs and melanoblasts cannot. The activity of soluble factor on mast cells and primitive haemopoietic progenitors *in vitro*[14-16] and on mast cells *in vivo*[9] is consistent with this idea.

Binding of tyrosine kinase receptors, such as c-*kit*, by their cognate ligands usually leads to activation of the kinase domain and transduction of signals that lead to cellular proliferation[17-19]. The MGF/c-*kit* complex mediates a proliferation response in mast cells[14,15,20] and, in combination with other factors, proliferation of primitive haemopoietic progenitors[11,15,16,20,21]. The data presented here and by Godin *et al.*[7] provide the first evidence of growth factor action on mouse PGCs and suggest a role for MGF in mediating a PGC survival signal rather than a proliferation signal. □

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1. Orr, U. A. *et al. Development* **109**, 911-923 (1990).
2. Keshet, E. *et al. EMBO J.* (in the press).
3. Matsui, Y., Zsebo, K. M. & Hogan, B. L. *Nature* **347**, 667-669 (1990).
4. Donovan, P. J., Stott, D., Cairns, L. A., Heasman, J. & Wylie, C. C. *Cell* **44**, 831-838 (1986).
5. Godin, I., Wylie, C. & Heasman, J. *Development* **108**, 357-363 (1990).
6. Cairns, L. thesis, Univ. Lond. (1987).
7. Godin, I. *et al. Nature* **352**, 807-809 (1991).
8. Copeland, N. G. *et al. Cell* **63**, 175-183 (1990).
9. Zsebo, K. M. *et al. Cell* **63**, 213-224 (1990).
10. Huang, E. *et al. Cell* **63**, 225-233 (1990).
11. Flanagan, J. G., Chan, D. C. & Leder, P. *Cell* **64**, 1025-1035 (1991).
12. Brannan, C. I. *et al. Proc. natn. Acad. Sci. U.S.A.* **88**, 4671-4674 (1991).
13. McCoshen, J. A. & McCallion, D. J. *Experientia* **31**, 589-590 (1975).
14. Williams, D. E. *et al. Cell* **63**, 167-174 (1990).
15. Anderson, D. M. *et al. Cell* **63**, 235-243 (1990).
16. Zsebo, K. M. *et al. Cell* **63**, 195-201 (1990).
17. Hanks, S. K., Quinn, A. M. & Hunter, T. *Science* **241**, 42-52 (1988).
18. Pawson, T. & Bernstein, A. *Trends Genet.* **6**, 350-356 (1990).
19. Yarden, Y. & Ullrich, A. *A. Rev. Biochem.* **57**, 443-478 (1988).
20. Nocka, K., Buck, J., Levi, E. & Besmer, P. *EMBO J.* **9**, 3287-3294 (1990).
21. Martin, F. H. *et al. Cell* **63**, 203-211 (1990).
22. Chomczynski, P. & Sacchi, N. *Analyt. Biochem.* **162**, 156-159 (1987).
23. Church, G. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1991-1995 (1984).
24. Dugaiczyk, A. *et al. Biochemistry* **22**, 1605-1613 (1983).
25. Graham, F. L. & Eb, A.v.d. *Virology* **52**, 456-467 (1973).
26. Boswell, H. S., Albrecht, P. R., Shupe, R. E., Williams, D. E. & Burgess, J. *Expl Hemato.* **15**, 46-53 (1987).

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Carboxy-terminal truncation activates *glp-1* protein to specify vulval fates in *Caenorhabditis elegans*

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THE *glp-1* and *lin-12* genes encode homologous transmembrane proteins[1,2] that may act as receptors for cell interactions during development[3,4]. The *glp-1* product is required for induction of germ-line proliferation and for embryogenesis[3,5]. By contrast, *lin-12* mediates somatic cell interactions, including those between the precursor cells that form the vulval hypodermis (VPCs)[6]. Here we analyse an unusual allele of *glp-1*, *glp-1(q35)*, which displays a semidominant multivulva phenotype (Muv), as well as the typical recessive, loss-of-function Glp phenotypes (sterility and embryonic lethality)[3]. We find that the effects of *glp-1(q35)* on VPC development mimic those of dominant *lin-12* mutations, even in the absence of *lin-12* activity. The *glp-1(q35)* gene bears a nonsense mutation predicted to eliminate the 122 C-terminal amino acids, including a ProGluSerThr (PEST) sequence thought to destabilize proteins. We suggest that the carboxy terminus bears a negative regulatory domain which normally inactivates *glp-1* in the VPCs. We propose that inappropriate *glp-1(q35)* activity can substitute for *lin-12* to determine vulval fate, perhaps by driving the VPCs to proliferate.

During wild-type development, three VPCs (P5.p, P6.p, P7.p) generate 22 descendants that form the vulval hypodermis (Figs 1 and 2*a*). Several genes are known that, when mutant, interfere with this process (reviewed in ref. 7). One of these is *lin-12*: animals with elevated *lin-12* activity (*lin-12(d)*) are Muv because all six VPCs adopt the VH2 fate[6]. The *glp-1* and *lin-12* genes

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RAPID COMMUNICATION

Kit Ligand in Synergy With Interleukin-3 Amplifies the Erythropoietin-Independent, Globin-Synthesizing Progeny of Normal Human Burst-Forming Units-Erythroid in Suspension Cultures: Physiologic Implications

By Thalia Papayannopoulou, Martha Brice, and C. Anthony Blau

Although the proliferative effects of hematopoietic cytokines on erythroid progenitors are well known, parameters that influence the initiation of expression of specialized or lineage-restricted genes are not clear. We have studied the acquisition of erythroid-differentiative features from enriched populations of human early erythroid progenitors (burst-forming unit-erythroid [BFUe]) in suspension culture and the influence of several cytokines on this process. In suspension cultures containing no erythropoietin (Epo), we have found that *kit* ligand (KL) in synergy with interleukin-3 not only increases the proliferation of cells and of progenitors but also consistently amplifies a population of cells that contain globin within 1 week. Our experiments suggest that neither extraneously provided nor endogenously produced Epo is critical for the generation of globin-synthesizing cells. Globin-producing cells generated mostly from late BFUe or pre-CFUe with a CD34⁻/EP-1⁺ phenotype in this system do not all express a well-coordinated erythroid program accompanied by heme or glycophorin A expression and most die maintaining an immature state. Therefore, conditions that are responsible for initiation of globin expression in these cells are not sufficient to carry them to terminal maturation. The data point to an expanded target cell population for KL, as they suggest an influence of KL on survival and/or amplification of late erythroid cells previously thought to be influenced only by Epo. Our results in aggregate are of relevance to the physiology of normal erythropoiesis and the role of Epo and KL in the initial stages of lineage-restricted gene expression. In addition, they provide insight into the understanding of anemia in W and Steel mutants in which expansion of the late erythroid progenitor pool, normally dependent on the synergistic action of KL and Epo, is curtailed.

HEMATOPOIETIC progenitors require a range of hematopoietic cytokines for their optimal development and terminal differentiation.[1-3] Interleukin-3 (IL-3), *kit* ligand (KL; or Steel factor, SCF, or MGF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) exert, especially in synergy, a profound proliferative influence on all hematopoietic progenitors, whereas several late acting cytokines (such as granulocyte-CSF [G-CSF] and erythropoietin [Epo]) are crucial for the maturation of specialized progeny from these progenitors. As pure recombinant factors are now available and highly enriched populations of hematopoietic progenitors can be obtained for testing, their growth requirements have been further refined.[4-15] Most of the information has been deduced using clonal assay systems for progenitors and has relied on the recognition of terminally differentiated progeny. Recently, in addition to these systems, suspension cultures have been used for the characterization of erythroid[16-19] or granulocytic progenitors.[19-23] These systems are easier to manipulate and have confirmed to a large extent the growth requirements of progenitors and the factors needed for their amplification. However, because the results so far have been evaluated in end-stage progeny of these progenitors and because the culture systems used may contain undefined as well as defined factors, the implementation of the early transition from the progenitor stage to the stage of lineage-specific gene expression has not yet been delineated.

As far as the erythroid system is concerned, Epo is considered the major trigger of coordinate expression of many or all of the erythroid-specific genes.[24,25] Nevertheless, both with human and murine cells, culture systems have been devised that appear to bypass the need for Epo throughout the entire maturation sequence of erythroid cells.[26-30] However, even in these systems, the factors responsible for the initiation of transcription of erythroid-specific genes versus the factors required for completion of the maturation process have not been defined. Furthermore, because of the pharmacologic concentrations of some of the factors used, it has been uncertain whether the in vitro data represent pathways operative in vivo.

To gain further insight into the sequence of events that are important during the early phases of erythroid progenitor (burst-forming unit-erythroid [BFUe]) differentiation and especially to identify factors that are needed to initiate the expression of specialized genes (ie, globin), we studied enriched populations of BFUe in a liquid suspension culture system, under conditions thought to promote or not to promote the generation of erythroid-differentiated progeny. In these studies, we focused our attention on changes within the first few days in liquid culture in an attempt to discover the initial decisions taken by the cells and the factors that may influence these decisions. We found that KL in synergy with IL-3 not only amplifies erythroid (and other) progenitors but it can influence the survival and/or the amplification of their descendants to the stage of globin-producing cells. However, these cells are unable to complete their maturation process and die prematurely in the absence of Epo. The data expand previous information pertaining to the physiology of normal erythropoiesis and to the role of KL in the early stages of expression of a specialized erythroid program.

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MATERIALS AND METHODS

Cells

Mononuclear cells recovered after centrifugation of platelet apheresis collections were used as a source of cells for the majority of the experiments (provided by Dr Thomas Price, Puget Sound Blood Bank, Seattle, WA). These cell preparations consisted primarily of small lymphocytes with a proportion of monocytes and a very small and variable contamination with granulocytes and red blood cells (RBCs). When RBC contamination was significant, the cells were treated for 2 minutes at 37°C with a hemolytic buffer (0.83% NH_4Cl, 0.1% $NaHCO_3$, 3.7 mg % EDTA) and washed with phosphate-buffered saline/bovine serum albumin (PBS/BSA; Dulbecco's Phosphate-Buffered Saline without calcium or magnesium; GIBCO, Grand Island, NY; with 1% BSA, Cohn Fraction V; Sigma, St Louis, MO, cat. no. A-4503). Cells were then resuspended in Iscove's modified Dulbecco's medium (IMDM; GIBCO) with 5% fetal calf serum (FCS; Gemini Bioproducts, Calabasas, CA) and incubated for 1 hour at 37°C on Falcon tissue culture plates to remove the majority of adherent cells. Nonadherent cells were washed off the plates and transferred for direct immunoadherence to anti-CD34 (12.8) or SR-1 antibody-coated plates, as described below. In two experiments, instead of mononuclear cells from apheresis, peripheral blood was drawn from normal volunteers. In three experiments, bone marrow aspirates from normal volunteers were used. All procedures were in accordance with institutionally approved protocols and with informed consent. Peripheral blood and aspirated bone marrow were subjected to density-gradient (1.077) centrifugation on a cushion of Hypaque-Ficoll (Lymphoprep, Nygaard, Oslo). Mononuclear cells collected from the interface were washed and incubated for the 37°C adherence incubation step as described above.

Growth Factors

Human recombinant growth factors were used at previously determined optimal concentrations in both suspension and clonal cell cultures. IL-3 (Genetics Institute, Cambridge, MA) was used at 50 U/mL; KL (SCF; Amgen, Thousand Oaks, CA) at 50 ng/mL; Epo (Genetics Institute) at 2 IU/mL; GM-CSF (Genetics Institute) at 45 ng/mL; and PIXY 321 (a GM-CSF/IL-3 fusion protein; Immunex, Seattle, WA) at 10 μg/mL.

Antibodies

The following monoclonal antibodies (MoAbs) were used: anti-CD34, either HPCA-1 (Becton Dickinson, San Jose, CA) or 12.8 purified protein (provided by I.D. Bernstein, Seattle, WA) or biotinylated 12.8 (provided by R. Berenson, CellPro, Bothell, WA); anti–*c-kit* receptor antibody SR-1[31] (provided by V.C. Broudy, Seattle, WA); the antierythroid progenitor/erythroblast antibody EP-1 (developed in our laboratory[32]); antiglycophorin (R-10; supplied by P.E. Edwards, London, UK); anti–IL-3 (a gift of K. Kaushansky, Seattle, WA); anti-Epo, polyclonal (Terry Fox Laboratories, Vancouver, BC, Canada); and the antiglobin antibodies (anti-β and anti-γ) and their fluorescent conjugates (all produced in our laboratory). As secondary antibodies, goat antimouse IgG (H + L), $F(ab)_2$-fluorescein isothiocyanate (FITC) (Hyclone, Logan, UT), or streptavidin-phycoerythrin (PE) (Biomeda, Foster City, CA) were used.

Surface Immunofluorescence

Live cells (about 5×10^4 or more) in PBS/BSA were incubated with primary antibodies (anti-CD34, SR-1, EP-1, and R-10) at 4°C for 20 minutes at a concentration of 1:100 or 1:200 for ascites or 5 to 25 μg/mL of purified antibody. Secondary antibody dilutions used varied from 1:20 to 1:50 depending on the lot. Treated cells were evaluated either with a Zeiss epifluorescence microscope (at 250× magnification) or by cytofluorometric analysis on an Ortho Cytofluorograf, model 50HH/2150 (Ortho Diagnostic Systems, Westwood, MA) or FACStar Plus (Becton Dickinson).

In addition, in one experiment, CD34 Ad cells were doubly labeled with anti-CD34 (12.8 biotinylated antibody followed by streptavidin-PE) and EP-1 antibody directly conjugated to FITC. These cells were analyzed and sorted with the FACStar Plus. Subsets that were either doubly positive ($CD34^+/EP\text{-}1^+$) or doubly negative ($CD34^-/EP\text{-}1^-$) for both antigens, or positive for only one of the two antigens ($CD34^+/EP\text{-}1^-$ or $CD34^-/EP\text{-}1^+$) were separated and used for further studies.

Antiglobin Labeling

The presence of β- or γ-globin was assessed by immunofluorescence on cytocentrifuge-prepared smears. These were fixed with methanol and treated with antiglobin MoAbs, as described previously.[33] Briefly, antibody at optimal concentrations in solution in PBS/BSA was placed in a standing drop on fixed, rinsed, and dried cytocentrifuge smears and incubated for 30 minutes at 37°C in a moist chamber. Between antibody steps and after antibody labeling, slides were rinsed sequentially in PBS and water, air-dried, and observed in a Zeiss epifluorescence microscope. Double labeling using directly conjugated anti-β or anti-γ antibodies conjugated with FITC or rhodamine was also performed, as described above. In addition to fixed cell smears, cells from suspension cultures and controls were fixed with methanol in suspension, labeled with optimal dilutions of anti-β or anti-γ MoAbs, and subjected to cytofluorographic analysis.

Globin Messenger RNA (mRNA) Analysis

Globin RNA analysis was performed by a slot-blot hybridization assay developed in our laboratory.[34] Briefly, cells from suspension culture (0.2 to 1×10^5) were washed twice in PBS and resuspended in cold TE buffer (10 mmol/L Tris, 0.1 mmol/L EDTA) to which 20 U of RNase inhibitor was added (RNAsin; Promega, Madison, WI). After lysing the cells with 5% NP-40, the lysates were centrifuged and transferred to tubes containing SSC (0.15 mol/L NaCl, 0.015 mol/L trisodium citrate) and formaldehyde (6× SSC and 8% formaldehyde), denatured at 65°C for 15 minutes, and used for slot blotting immediately or stored at −70°C for later analysis.

For detection of β-globin mRNA, the 0.7-kb *Pst*I/*Eco*RI fragment containing the 3′ end of the human genomic β-globin was subcloned in the antisense orientation in T7 plasmid. For γ-globin mRNA, the 0.6-kb *Eco*RI/*Hin*dIII fragment of the 3′ end of Gγ-globin gene was subcloned in a Sp6 vector. Hybridization conditions were as described[34] and the relative amounts of γ- and β-globin mRNAs were calculated after phosphoimager analysis.

Benzidine Test

To assess heme content in cells, we used a sensitive (wet) benzidine assay as previously described[35] or the method commonly used for fixed cell smears or fixed plasma clots.[36]

Immunoadherence ("panning")

Immunoadherence of cells to Petri dishes precoated with MoAbs has been previously described by ourselves and others.[37,38] In the studies described here, Petri dishes were precoated with MoAb, either purified anti-CD34 (12.8) at 25 μg/mL or anti–*c-kit* (SR-1) ascites at 1:200 dilution, in 0.05 mol/L Tris buffer, pH 9.5, at room temperature for 45 minutes. Untreated cells in PBS/BSA were incubated directly on the panning plates for 1 hour at 4°C. After washing the nonadherent cells from the plates with cold PBS/BSA, adherent cells were incubated for 20 to 30 minutes at 37°C to loosen specifically adhering cells. Adherent cells were then recovered by flushing the plates with warm IMDM/FCS. "CD34 Ad" or "SR-1 Ad" cells were

incubated overnight at 37°C followed by, if enough cells were available, a second round of direct (anti-CD34 or SR-1) panning to further increase the specificity of adherence. Nonadherent cells from this second step, as well as adherent cells, were recovered and used for clonal or suspension cultures as described below.

Cell Cultures

Liquid suspension cultures. After one or two rounds of direct panning, enriched progenitor populations were incubated at 37°C in stationary suspension culture in conical 15 mL polystyrene centrifuge tubes placed at a 25° angle. From 1 to 2 × 10^5 cells/mL in 1 or 2 mL aliquots were cultured for 6 to 14 days. Eighty percent to 90% of the medium and additives were replaced every 48 hours. When aliquots of cells were removed for sampling, this was performed after mixing and replacing the medium, and cell determinations were based on per unit volume at each test day. The basic suspension medium was either serum-containing or serum-free. Serum-containing culture medium included 20% FCS and 10% human AB serum from normal volunteers (both heat-inactivated), 1% BSA (Cohn Fraction V; Calbiochem, San Diego, CA), 10^{-4} mol/L 2-mercaptoethanol (Eastman Kodak, Rochester, NY), and IMDM. Serum-free medium was composed of 90% Ex-cell 300 defined culture medium (JRH Bioscience, Lenexa, KS) supplemented with 1% BSA, iron-saturated human transferrin (early experiments were performed without additional supplementation with transferrin, relying only on the small amount of transferrin present in the Ex-cell 300), a mixture of nucleosides (each at a final concentration of 10 μg/mL), 10^{-4} mol/L 2-mercaptoethanol, and L-glutamine. Growth factors or antibodies were added to these media in microliter amounts per milliliter.

Clonal cultures. Peripheral blood or bone marrow cells prepared as described above were cultured after each stage in the purification sequence (ie, before and after panning).[38] Methylcellulose culture media contained the same components as the serum-supplemented suspension cultures with the addition of 1% methylcellulose (Fischer Scientific, Pittsburgh, PA). Plasma clot culture medium contained the same concentrations of FCS, AB serum, BSA, and 2-mercaptoethanol as the methylcellulose medium, but with the addition of bovine citrated plasma (Irvine Scientific, Irvine, CA) and beef embryo extract (GIBCO), each added at 10%, instead of methylcellulose. To these media the cytokines IL-3, KL, GM-CSF, and Epo, and the antibodies anti-Epo, anti-IL-3, and anti-c-*kit* (SR-1) were added alone or in various combinations. Colonies of different types were identified by morphologic criteria: colony-forming unit-erythroid (CFUe) in plasma clot cultures at days 5 to 7; BFUe, colony-forming unit-granulocyte and/or macrophage (CFU-GM), and CFU-Mix (one or more lineages in addition to erythroid) in live methylcellulose cultures at days 11 to 14. In the live cultures, BFUe colonies were large hemoglobinized, often multisegmented, and often macroscopic; CFU-GM were compact or loose colorless colonies; CFU-Mix colonies were large, sometimes multi-unit aggregates with both hemoglobinized and nonhemoglobinized portions. CFUe colonies were evaluated in flattened whole mounts of individual plasma clots fixed with glutaraldehyde and stained with benzidine and hematoxylin; they were defined as mature hemoglobinized, small compact clusters of 8 to 60 cells.

RESULTS

Enrichment of Circulating Hematopoietic Progenitors by a Positive Immunoadherence Step

The adherent cells recovered after the first positive immunoadherence of peripheral mononuclear cells on anti-CD34- or SR-1-coated plates represented approximately 1.2% to 2% of the initial cell population; adherent cells recovered after a second direct panning with the same antibodies were from 0.03 to 0.2% of the original cells. The cells from three normal bone marrow mononuclear cells were panned once and were on the average 2% of the initial cells.

Cells selected by panning with either anti-CD34 or SR-1 appeared as small lymphocytes with a variable proportion of larger blast-like cells or monocytes. Positivity of "CD34 Ad" cells labeled with an anti-CD34 MoAb recognizing a different epitope (HPCA) from the one used for panning (12.8) was found to be from 40% to 70%. Positivity of "SR-1 Ad" cells was tested the day after panning with the SR-1 antibody, because an antibody to a different epitope was not available. The percent positive cells averaged 30% in three experiments when the total population was analyzed, but was greater than 80% when only the larger blast-like cells were gated.[38]

To assess the frequency of clonogenic progenitors in the "CD34 Ad" or "SR-1 Ad" cells, we cultured them in clonal methylcellulose cultures under optimal conditions in the presence of IL-3, KL, GM-CSF, and Epo. The frequency of BFUe in these panned cells is shown in Table 1. In nine experiments using "SR-1 Ad" cells, the cloning efficiency of BFUe was from 2% to 27%, with a mean of 9.5% ± 3.4% SEM, whereas frequencies before panning were from 0.02% to 0.44%. The frequency of BFUe in "CD34 Ad" cells in 16 experiments was from 5% to 24.6%, with a mean of 11.3% ± 1.5% SEM. (Frequencies in CD34 nonadherent or SR-1 nonadherent cells were much lower than in unseparated cells.) Large differences in enrichment among experiments may represent both biologic differences and possible differences in cell binding depending on whether ascites or purified antibody was used. Whenever two rounds of panning were used, enrichment improved, at the expense, however, of total progenitor yield (data not shown). Nevertheless, we were surprised to find that the progenitor purification and yield using only two rounds of direct panning without preliminary en-

Table 1. Frequency of BFUe in Cells Selected by Immunoadherence With Anti-c-*kit* or Anti-CD34 Antibodies

BFUe Percent	
Anti-CD34 "Panned"	Anti-c-*kit* "Panned"
9.7	5.5
5.1	3.1
15.7	24.8
6.3	1.5
13.9	15.0
1.7	4.7
18.2	27.0
10.7	1.1
4.7	3.9
7.1	
9.7	
20.2	
24.6	
9.3	
5.1	
8.6	
9.5 ± 3.4 SEM	11.3 ± 1.5 SEM

richment of progenitors was comparable to those reported in previous studies using preliminary negative enrichment steps or FACS sorting.[5,11,14,39] The bursts from day 0 enriched cells were large, and a high proportion (about a third of the total) were macroscopic, containing over 10,000 cells. No CFUe-like colonies were seen in cultures from either peripheral blood or bone marrow "CD34 Ad" or "SR-1 Ad" cells. No hemoglobinized colonies were usually observed in the absence of Epo in these cultures. Recovery of original BFUe in the adherent cell samples (calculated as numbers of BFUe in the "panned" population divided by the number of BFUe in the original population) showed a wide variation (range, 2% to 70%), but was usually above 15%.

Cell Proliferation and BFUe Amplification in Short-Term Liquid Cultures: The Effect of Cytokines

Cell proliferation was assessed by cell counts in all suspension culture experiments on days 6 and 7 and up to 2 weeks in certain experiments. In the absence of growth factors, no cell growth was noted compared with the input number of cells (data not shown). However, in the presence of IL-3 and KL, there was a significant increase in cell number over a 6-day period, of up to 18-fold among the 19 experiments performed. In the presence of a single cytokine, ie, IL-3 or KL or Epo, there was less growth (up to 4-fold) during the same 6- to 7-day period.

The number of progenitors present after 1 week in suspension culture was assessed by secondary plating in clonal methylcellulose culture. The frequency of BFUe (after 6 days in suspension) ranged from 1% to 53% (Table 2). Highest frequencies again were observed after suspension culture in IL-3 and KL compared with either of these cytokines alone. BFUe amplification at day 6 over the number at day 0 was from threefold to 40-fold, with a mean of 11-fold ± 2.7-fold SEM and occurred in all cultures containing combinations of cytokines (Fig 1). Such increments in cell proliferation and progenitor amplification are in keeping with prior studies of enriched progenitors from bone marrow or peripheral blood from our own and other laboratories.[5,11,14,38]

Morphologic Changes and Generation of Globin(+) Cells in Suspension Cultures of Enriched Progenitors

Cultures containing cytokines but excluding Epo. In addition to testing cell proliferation and progenitor amplification by cell counting and clonal assays for progenitors, cytospin preparations were prepared routinely after 1 week in suspension culture and in selected experiments on days 2, 4, 6, 10, and 12. Preparations from cultures without cytokines showed many unhealthy cells and minimal differentiation. However, cultures containing cytokines, especially those containing IL-3 and KL, showed healthy populations of immature blasts ranging from 20% to more than 50%, depending on the experiment (Fig 2). The small lymphoid cells seen at day 1 have greatly diminished or virtually disappeared by day 6. In addition to blast-like cells, immature cells with basophil-like granules were present (Fig 2) in Wright's-Giemsa-stained preparations. The same populations were also tested for benzidine positivity (by the wet benzidine method) and for the presence of β- or γ-globin using monoclonal anti-β and anti-γ antibodies, as described in Materials and Methods. Wet benzidine staining, always negative in day 0 to 1 preparations, ranged from 1% to 20% at days 6 to 7 (Table 3). Benzidine positivity in dried smears was always much lower or totally negative (data not shown). Frequencies of anti-β or anti-γ

Table 2. Effect of Anti–c-*kit* (SR-1) or Anti–IL-3 Antibody on BFUe and CFU-GM (number and percentage of total cells) and on β-Globin (+) Cells (number and percentage of total cells) Generated From 1 × 10^5 Input "CD34 Ad" Cells After 1 Week in Suspension Culture in Serum-Containing (S) or Serum-Free (SF) Media

Experiment No.	Additives*	CFUe† No.	CFUe† (%)	BFUe† No.	BFUe† (%)	CFU-GM† No.	CFU-GM† (%)	β-Globin (+) No.‡	β-Globin (+) (%)
1 (S)	Epo	600	(0.8)	2,100	(0.3)	75	(0.01)	638,250	(85.1)
	Epo + SR-1	560	(0.08)	140	(0.02)	0	(0.0)	640,500	(91.5)
	IL-3 + KL	4,290	(0.8)	29,700	(5.4)	825	(0.15)	286,000	(52.0)
	IL-3 + KL + SR-1	34	(0.02)	306	(0.2)	204	(0.12)	1,700	(1.0)
2 (SF)	IL-3 + KL	15,391	(6.3)	51,180	(21.0)	810	(0.3)	47,450	(19.5)
	IL-3 + KL + SR-1	5,308	(2.4)	17,117	(7.9)	650	(0.3)	6,283	(2.9)
3 (S)	IL-3	ND	ND	16,400	(2.7)	2,340	(0.4)	9,600	(1.6)
	IL-3 + SR-1	ND	ND	4,770	(1.1)	900	(0.2)	3,465	(0.8)
4 (SF)	IL-3 + KL	50,721	(3.2)	490,622	(30.8)	10,208	(0.6)	172,260	(10.8)
	IL-3 + KL + αIL-3	27,136	(2.8)	519,622	(52.7)	5,522	(0.6)	139,026	(14.1)
5 (S)	KL	2,829	(1.3)	11,022	(5.0)	242	(0.1)	24,200	(11.0)
	KL + αIL-3	5,163	(3.2)	15,400	(9.7)	118	(0.07)	29,760	(18.6)
	Epo	29,940	(14.3)	23,310	(11.1)	1,029	(0.5)	160,650	(76.5)
	Epo + αIL-3	25,297	(6.7)	19,418	(5.1)	1,596	(0.4)	215,460	(56.7)

Abbreviation: ND, not done.
*Present in suspension medium only; replating (colony culture) conditions were the same for all samples (see Materials and Methods).
†Assessed by replating cells from suspension cultures into plasma clot (CFUe) or methylcellulose (BFUe and CFU-GM) cultures.
‡Calculated from the frequency of β-globin (+) in smears and the total number of cells in liquid culture on the same day.

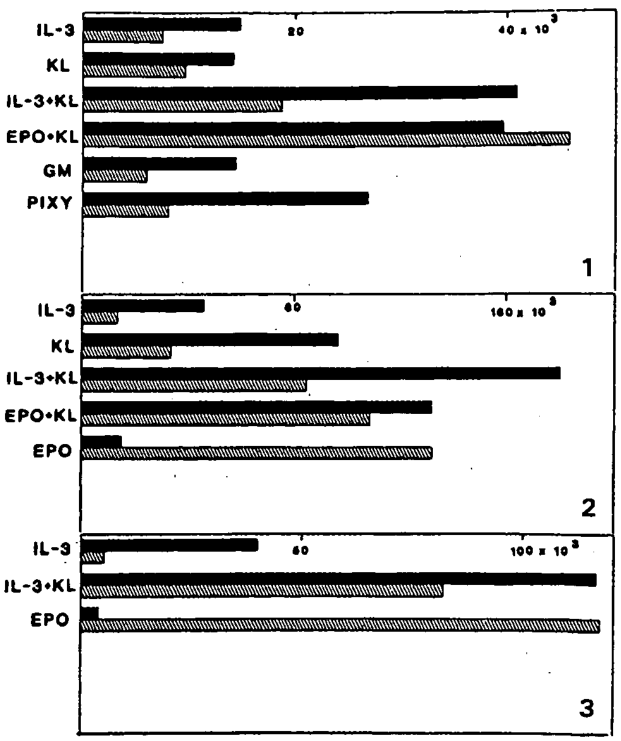

Fig 1. Number of BFUe (■) and globin(+) cells (▨) in suspension cultures of immunoadherence-enriched ("CD34 Ad") peripheral blood cells (10^5 input cells) after 1 week in the presence of cytokines (IL-3, KL, GM, and PIXY 321) without Epo or in the presence of Epo alone or with Epo + KL. (Results from three independent experiments: #1 and #2 with serum and #3 without serum.) The total number of BFUe was highest in the combination IL-3 + KL. In the absence of Epo, the total number of newly generated globin(+) cells was also highest with the combination of IL-3 + KL.

globin-positive cells are shown in Tables 2 through 4 and Figs 3 and 4. In 14 experiments, they ranged from 4% to 38% at day 6 to 7, and the frequency of BFUe in the same cell populations was from 4% to 53% (Tables 2 through 4). Thus, cultures containing IL-3 and KL contained a large proportion of erythroid cells at the BFUe level and at the globin-synthesizing cell level. Surface labeling of these populations was performed in selected experiments using anti-CD34, SR-1, EP-1, and antiglycophorin A antibodies. A significant decrease (more than two-thirds) in both CD34- and SR-1-positive cells was observed at day 7 compared with day 0 (data not shown). In contrast, EP-1 positivity increased from about 5% to 10% at day 0 to greater than 50% at day 6. Glycophorin A was negative at day 0 in "CD34 Ad" cells prepared from either peripheral blood or bone marrow, but these cells became 15% to 33% positive at day 6 to 7. Frequency of glycophorin A-positive cells was usually less (by one-third to one-fifth) than the frequency of globin(+) cells. When IL-3 or KL were each used alone, not only was there less amplification in cell numbers and in progenitors, but also the number of globin(+) cells generated were greatly diminished (Fig 1).

To gain further insight into the synergistic action of IL-3 or KL when they were used in combination, we performed experiments in which we used anti–c-*kit* receptor antibody (SR-1). The results of these experiments are shown in Table 2. The presence of SR-1 in suspension culture dramatically reduced not only cell proliferation and amplification of BFUe but also the generation of β-globin(+) cells. In previous experiments, we showed that the addition of SR-1 to Epo- or IL-3–containing clonal cultures profoundly reduced the number of erythroid bursts,[31,38] implying that c-*kit* receptor function in the context of IL-3 or Epo is very important for BFUe development. This conclusion was later independently confirmed by other approaches.[40,41] Here we show in addition that *kit* ligand/receptor interactions are crucial for the generation of globin(+) cells in suspension culture. The presence of anti–IL-3 antibody in cultures containing IL-3 and KL did not diminish the effectiveness of KL, although complete neutralization of IL-3 was not proven in these experiments. Furthermore, the action of KL or Epo alone in suspension cultures did not seem to be influenced by putative endogenously produced IL-3 (Table 2).

Cultures containing Epo. The morphology of cells incubated in the presence of Epo was in striking contrast to that of cells incubated with IL-3 and KL. After 1 week in culture, a population of healthy basophilic blasts resembling proerythroblasts was present together with cellular debris. There were no cells containing granules as seen in IL-3 + KL cultures. The effects on cell proliferation, on progenitor amplification, and on generation of globin(+) cells are shown in Tables 2 through 4 and Figs 1, 3, and 4. The combination of Epo and KL was comparable to IL-3 and KL, ie, very effective both in terms of cell proliferation and expansion of BFUe (Fig 1 and data not shown). All preparations containing Epo generated the most globin(+) cells both in absolute numbers and in relative frequency (Figs 1 and 3, and Table 2).

Another major difference between Epo versus IL-3 + KL cultures was found in suspensions examined beyond the first week in culture. During the second week, the frequency of

Fig 2. Morphologic appearance of cells ("CD34 Ad") after 1 week in suspension culture in the presence of IL-3 + KL. Note the presence of basophilic blasts (dark cytoplasm) and of cells containing granules. The latter were absent in Epo-only cultures, in which mainly the dark basophilic cells were present along with a high proportion of "dying" cells.

Table 3. Influence of Incubation Medium (serum [S] v Serum Free [SF]) on BFUe Content, Proportion of γ- and β-Globin (+) Cells and Benzidine (+) Cells (by the wet benzidine method) After 7 Days in Suspension Culture (of 1 × 10⁶ "SR-1 Ad" or "CD34 Ad" cells)

Experiment No.	Medium	Cytokine*	BFUe (no.)†	Globin (+) Cells γ No.‡	γ (%)	β No.‡	β (%)	Benz + (%)
1 ("SR-1 Ad")	S	Epo	19,305	42,236†	(20.2)	112,490†	(53.8)	47.6
	S	IL-3 + KL	36,157	54,264	(10.4)	71,091	(13.6)	8.4
	SF§	IL-3 + KL	37,800	5,760	(0.8)	60,480	(8.4)	0.8
2 ("SR-1 Ad")	S	Epo	1,003	11,286	(17.1)	27,522	(41.7)	
	SF§	Epo	880	1,188	(3.6)	4,488	(13.6)	
3 ("CD34-Ad")	S	IL-3 + KL	52,000	19,000	(1.9)	61,000	(6.1)	
	SF	IL-3 + KL	116,220	6,760	(0.5)	81,900	(6.3)	
4 ("CD34-Ad")	S	IL-3 + KL	51,180	21,413	(8.8)	47,450	(19.5)	7.7
	SF	IL-3 + KL	55,680	2,707	(2.8)	33,930	(35.1)	21.0
	S	Epo	18,675	47,813	(32.6)	87,413	(59.6)	63.0
	SF	Epo	20,457	10,640	(5.6)	142,120	(74.8)	66.0
5 ("CD34-Ad")	S	Epo	15,365	42,840	(30.0)	132,378	(92.7)	
	SF	Epo	12,153	13,923	(11.7)	109,837	(92.3)	
	S	Epo + KL	132,480	53,645	(16.1)	108,956	(32.7)	
	SF	Epo + KL	84,066	25,978	(5.9)	241,284	(54.8)	

*In suspension culture only; replating (colony culture) conditions were the same for all samples.
†See Table 2.
‡See Table 2.
§Different SF than in experiments 3 through 5.

globin(+) cells declined in IL-3 + KL cultures (Fig 3) without any evidence of further maturation. In contrast, globin(+) cells in Epo-containing cultures increased to more than 90% (Fig 3) by day 13 to 14, accompanied by an accumulation of smaller, more mature, strongly benzidine(+) cells. In a few experiments, in addition to anti–β-globin labeling, cytospin preparations from six cultures (with or without Epo) were doubly labeled with an anti–γ-globin antibody. Cells positive for γ-globin were also positive for β-globin, and therefore did not represent a separate population. Overall, the proportion of benzidine(+) cells was lower than that of β-globin(+) cells; this was especially true in serum-free medium and in the absence of Epo.

Liquid Cultures in Serum Versus Serum-Free Medium

In five experiments we compared the results of combinations of cytokines and Epo in the presence or absence of serum. Under serum-free conditions, cell proliferation and BFUe amplification were comparable to serum-containing cultures and there were no significant differences in the generation of β-globin(+) cells (Table 3). However, replicate

Table 4. Effect of Anti-Epo Antibody on BFUe or CFU-GM Content and Generation of β-Globin (+) Cells From 10⁶ "CD34 Ad" Cells Cultured in Serum (S) or Serum-Free (SF) Liquid Medium for 1 Week

Experiment No.	Additives*	CFUe† No.	CFUe† (%)	BFUe† No.	BFUe† (%)	CFU-GM† No.	CFU-GM† (%)	β-Globin (+) No.‡	β-Globin (+) (%)
1 (S)	Epo	3,960	(2.7)	18,627	(12.7)	245	(0.2)	87,413	(59.6)
	Epo + anti-Epo	420	(0.6)	6,563	(8.9)	140	(0.2)	3,220	(4.6)
2 (S)	None	119	(0.04)	2,467	(0.9)	967	(0.3)	<162	(0)
	Epo	1,681	(0.5)	8,014	(2.2)	954	(0.3)	65,520	(18.2)
	Epo + anti-Epo	0	(0)	2,597	(0.7)	1,253	(0.4)	<350	(<0.1)
	IL-3 + KL	3,824	(1.0)	11,444	(2.8)	3,340	(0.8)	10,800	(2.7)
	IL-3 + KL + anti-Epo	1,306	(0.3)	4,675	(1.1)	1,949	(0.5)	13,440	(3.2)
3 (SF)	IL-3 + KL	ND	ND	58,451	(11.3)	21,245	(4.1)	164,782	(31.8)
	IL-3 + KL + anti-Epo	ND	ND	43,789	(10.4)	20,632	(4.9)	83,368	(19.8)
4 (SF)	IL-3 + KL	50,721	(3.2)	490,622	(30.8)	9,570	(0.6)	172,260	(10.8)
	IL-3 + KL + anti-Epo	43,778	(3.0)	579,176	(39.2)	11,832	(0.8)	158,253	(10.7)

Abbreviation: ND, not done.
*See Table 2.
†See Table 2.
‡See Table 2.

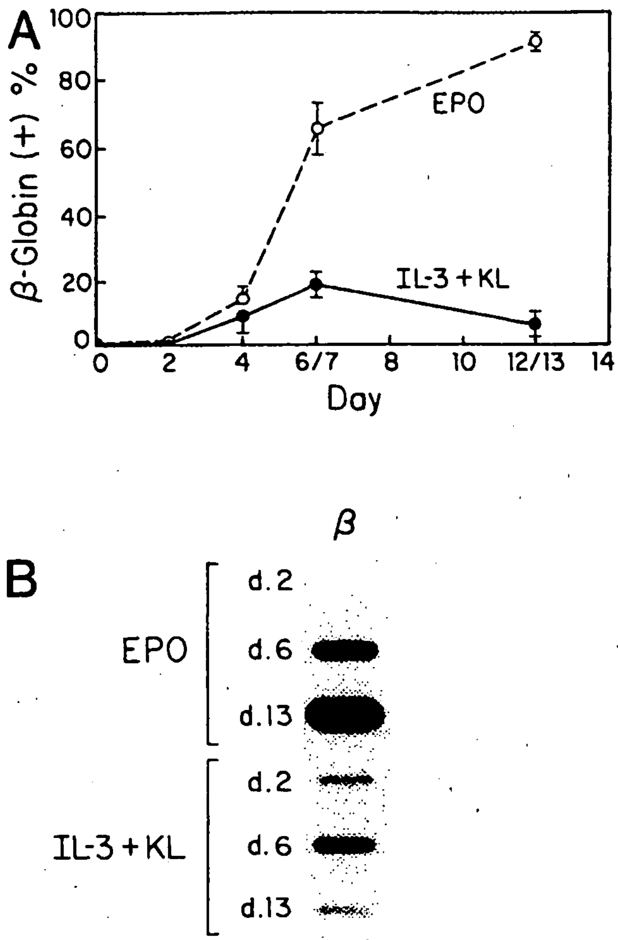

Fig 3. (A) Proportion of β-globin (+) cells over time in suspension cultures containing either IL-3 + KL (●) or Epo only (○). Whereas β-globin(+) cells increase and mature with time in culture containing Epo, β-globin(+) cells in IL-3 + KL reach their peak at 6 to 7 days and die retaining their immature state. Each point represents the mean (±SEM) of 4 to 14 experiments. (B) β-globin mRNA over time in suspension cultures in one representative experiment (same number of cells were used for all samples). Note the decline in β RNA between day 6 and day 13 in the absence of Epo, whereas the opposite is observed (ie, increase in β-globin mRNA) in Epo-containing samples.

preparations stained with anti–γ-globin showed a consistent and significant decrease in the proportion of γ-globin cells in serum-free conditions compared with serum-containing ones, with all combinations of cytokines tested (IL-3, KL, or Epo). Thus, the effect of serum-free conditions on γ-globin synthesis noted in previous clonal cultures[34,42,43] appears to be confirmed here and to be dominant regardless of the combination of cytokines used. For example, no consistent trend in proportion of γ-globin was found when cultures containing KL (at 50 μg/mL) were compared with cultures containing only Epo. The differences in γ-globin (between serum-containing and serum-free cultures) seen with fluorescent-labeled antiglobin antibodies were confirmed at the mRNA level in slot-blot hybridization assays (Fig 5). In cultures without serum, γ-globin mRNA was either very low or barely detectable compared with β-globin mRNA.

Experiments With Anti-Epo

The above results with serum-free cultures suggested that Epo is not critical for the generation of globin(+) cells in IL-3 + KL-containing cultures (Table 3). To further pursue whether any endogenous Epo was present in the system, we performed three experiments in which anti-Epo neutralizing polyclonal antibodies were included in the serum-free culture medium in the presence and in the absence of Epo. In clonal culture controls, these antibodies were found to neutralize the Epo effect, as no mature bursts were observed in meth-

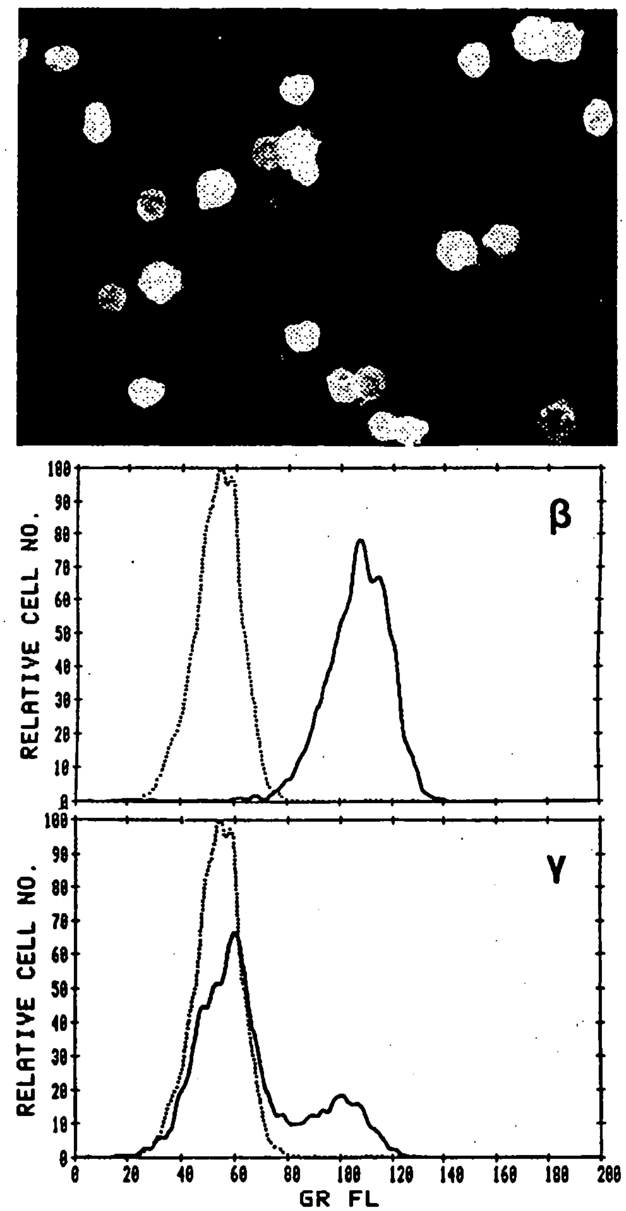

Fig 4. Antiglobin labeling of cell populations enriched in progenitors ("CD34 Ad") after 1 week in suspension culture in the presence of IL-3 + KL (top, fixed and stained smear) or Epo only (FACS analysis, middle and bottom). In IL-3 + KL (top), about 30% of cells were positive with β-globin, whereas in Epo (middle) the great majority (>95%) were β-globin(+). A smaller proportion of cells (14%) had γ-globin (bottom).

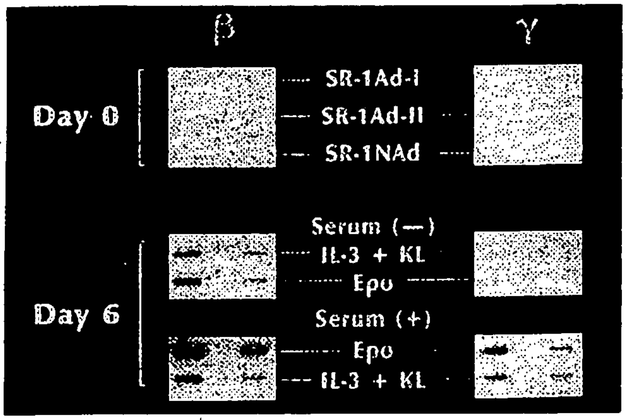

Fig 5. β- and γ-globin mRNA in "CD34 Ad" cells before (day 0) and after (day 6) culture in suspension. Note the difference in γ-globin mRNA in serum (−) compared with serum (+) conditions.

ylcellulose cultures in the simultaneous presence of Epo and anti-Epo (data not shown). Anti-Epo was added to suspension cultures on day 1 (at concentrations sufficient to overcome three times the level of Epo used), and fresh medium containing anti-Epo was added every 2 days for 1 week. The amplification in BFUe and CFU-GM and the generation of globin(+) cells was assessed in the presence of anti-Epo (in suspension cultures) with several cytokine combinations. When anti-Epo was added to suspension cultures containing Epo only, there was a drastic reduction in the expansion of BFUe (assessed by replating cells in Epo-containing clonal cultures) and in the generation of β-globin(+) cells, to levels observed without any growth factor (Table 4). When anti-Epo was added to cultures containing IL-3 and KL, there were no significant effects in serum-free cultures, although some effect was noted in serum-containing cultures (Table 4). In the presence of serum, in addition to a decrease in BFUe, a reduction in the amplification of CFU-GM was found; this could be explained at least in part as a nonspecific effect of the antibody (a rabbit polyclonal antiserum) on progenitor clonal development.

Experiments With Anti-CD34 and EP-1 Doubly Labeled Cells

Previous experiments[32] have shown that EP-1 antibody reacts preferentially with erythroid (BFUe) rather than nonerythroid progenitors. To study the nature of the immediate precursors of the globin(+) progeny in our suspension cultures and/or to enrich for these precursor cells, we doubly labeled the CD34 Ad cells with anti-CD34 and EP-1 the day after panning. The proportion of each subset (+/+, −/+, +/−, −/−) in the total population was recorded; aliquots of each subset were immediately plated in clonogenic progenitor assays and in suspension culture in the presence of IL-3 and KL. After 6 days in suspension, cell numbers, frequency of clonogenic progenitors, and frequency of globin(+) cells were determined. As shown in Table 5, although the $CD34^-/EP\text{-}1^+$ subset represented only 15% of the total population, it had the highest frequency of BFUe at day 0. This subset not only maintained the highest frequency of BFUe and CFUe in suspension culture, but, most impressively, led to the highest total number (and highest frequency) of globin(+) cells in suspension culture, suggesting that $CD34^-/EP\text{-}1^+$ cells are the immediate progenitors of most globin(+) cells in unsorted populations. The doubly positive subset ($CD34^+/EP\text{-}1^+$) gave rise to less BFUe than the previous subset, but these were larger in size and appeared to have expanded more during the 6-day period in suspension culture than those from the $CD34^-/EP\text{-}1^+$ cells. These data are consistent with the notion that $CD34^+/EP\text{-}1^+$ are more primitive erythroid progenitors than $CD34^-/EP\text{-}1^+$. The subset that was least positive or negative for EP-1 but still positive for CD34 ($CD34^+/EP\text{-}1^-$) appeared to have the highest proportion of CFU-GM (9%); these were greatly expanded by day 6 to constitute 15% of the whole population, in contrast to all other subsets that

Table 5. CD34 Ad Cells Labeled With Anti-CD34 and EP-1 Antibodies and Separated by FACS Into Doubly Positive ($CD34^+/EP\text{-}1^+$), Singly Positive ($CD34^-/EP\text{-}1^+$; $CD34^+/EP\text{-}1^-$), and Doubly Negative ($CD34^-/EP\text{-}1^-$) Subsets

	BFUe		CFUe		Globin (+) Cells			
					γ		β	
	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6	Day 0	Day 6
$CD34^+/EP\text{-}1^+$ (24%)	3,538 (8.8%)	47,850 (3.2%)	ND	18,750 (1.3%)	0	24,000 (1.6%)	0	42,000 (2.8%)
$CD34^-/EP\text{-}1^+$ (15%)	10,360 (24.6%)	63,996 (5.3%)	ND	23,196 (1.9%)	0	81,600 (6.8%)	0	182,400 (15.2%)
$CD34^+/EP\text{-}1^-$ (42%)	3,620 (7.5%)	26,660 (1.3%)	ND	2,340 (0.1%)	0	1,400 (0.07%)	0	4,000 (0.2%)
$CD34^-/EP\text{-}1^-$ (19%)	1,340 (3.0%)	4,056 (3.1%)	ND	ND	0	65 (<0.05%)	0	520 (<0.4%)

Concentration and amplification of erythroid progenitors (BFUe, CFUe) is shown in the different subsets before and after 6 days in suspension culture (cultures were initiated at day 0 with 4 to 5×10^4 cells in the presence of IL-3 + KL). The number and proportion of globin (+) cells (γ, β) is also shown. The $CD34^-/EP\text{-}1^+$ subset yielded the highest number of globin (+) cells. Numbers in parentheses indicate proportion of each subset in the total population.

Abbreviation: ND, not done.

had less than 1%. BFUe present in this subset were nearly all (96%) of large size. Collectively, these data provide new information about the phenotypic heterogeneity of BFUe and suggest a developmental hierarchy, from $CD34^+/EP\text{-}1^-$ to $CD34^+/EP\text{-}1^+$ to $CD34^-/EP\text{-}1^+$.

DISCUSSION

In the studies presented here, we have focused our attention on the detection and evaluation by sensitive approaches of newly acquired erythroid differentiation features when enriched populations of erythroid progenitors are placed in suspension culture under the influence of various hematopoietic cytokines. Specifically, the effects of early cytokines, such as KL or IL-3, were studied and the data were compared with control cultures containing Epo. The major findings from our studies are: (1) the consistent generation from normal BFUe of a significant number of Epo-independent globin-synthesizing cells during 1 week in suspension culture; (2) KL in synergy with IL-3 is instrumental in the accumulation of these globin-containing cells, suggesting that it can influence cells previously thought to be exclusively influenced by Epo; (3) globin appears to be an earlier rather than a later marker of erythroid differentiation and conditions that initiate its synthesis in cultures without Epo are not sufficient alone to carry the cells through their terminal maturation.

Several lines of evidence in our work suggest that neither extraneously provided nor endogenously produced Epo is critical for the formation of the globin synthesizing BFUe progeny. Results of experiments performed in serum-free, chemically defined media or in the presence of neutralizing anti-Epo antibodies argue against the need for the presence of Epo in our system (Tables 3 and 4). However, the above data do not exclude the possibility of an autocrine regulation by a certain class of erythroid progenitors (CFU-Mix), as suggested by a recent report.[44] Preliminary experiments using our culture system with the enriched CD34 Ad population rather than the unpurified bone marrow cells used in the previous report have failed to detect, by reverse transcription-polymerase chain reaction (RT-PCR) or RNase protection assays, any Epo mRNA either at day 0 or after suspension culture for 1 week with IL-3 and KL. Furthermore, using the same antisense strategy used in the previously published work[44] with our culture system (with two sense and two antisense oligonucleotides, freshly renewed every 48 hours in serum-free cultures), we failed to uncover differences between sense- and antisense-containing cultures (data not shown).

Our results, ie, the regeneration of Epo-independent globin-synthesizing cells in suspension, are at variance with several previous reports using suspension cultures supplemented with hematopoietic cytokines, in which no detectable erythroid differentiation was seen in the absence of added Epo.[16-19,22,23] Their conclusions were based on the absence of benzidine positivity or absence of glycophorin A positivity. However, these results may not necessarily be contradictory to our own but instead may be attributable to differences in the sensitivity of the approaches used (ie, globin positivity versus benzidine or glycophorin A positivity) and to the fact that we have used enriched progenitor populations.

Although our demonstration of globin(+) cells in suspension cultures lacking Epo is novel, in clonal cultures the development of erythroid characteristics under conditions of Epo deprivation has been documented by several laboratories. For example, this result is observed routinely with cells from patients with myeloproliferative syndromes[45-48] and to a lesser extent in situations with expanded erythropoiesis.[49-51] Furthermore, under special conditions, hemoglobinized colonies can develop from normal progenitors if specific additives, such as IGF-I, IGF-II, hemin, or retinoids (usually in pharmacologic doses) are used instead of Epo.[30] There are major differences between this previous clonal culture system using normal individuals[30] and our own suspension system. First, no special components were used in our cultures, ie, no IGF, hemin, or retinyl-acetate. In fact, our serum-free conditions were so minimal that the same medium used in methylcellulose clonal cultures was inadequate for clonal growth of the same progenitors. Second, in contrast to this previous report,[30] accessory cells were not needed in our suspension culture system. Furthermore, whereas our system does not support the full maturation of erythroid cells in the absence of Epo, we are able to identify them early without the need for color (heme) formation. Despite the above differences, however, a common denominator between our suspension system and previous clonal cultures (with either normal murine[26-29] or normal human[30] progenitors) was the presence of cytokines, either in purified form or in the form of conditioned media. It would appear that the presence of cytokines (IL-3 and KL) in the absence of Epo is important for the early stages of erythroid-specific gene expression (ie, globin synthesis) and for formation of the cells we are detecting, whereas other factors (such as Epo, insulin, activin, hemin, or IGF-I and II) are needed for enhancing their further maturation.

In addition to their cytokine requirements, questions pertaining to the identity, the state of differentiation, and the fate of these globin(+) cells in suspension culture were addressed in our studies. By morphologic criteria, these globin(+) cells, with immature nucleus and deep basophilic cytoplasm with perinuclear clear zone, resemble cells at the CFUe/proerythroblast level.[14] However, we do not think that these cells are descendants of CFUe; instead, we believe that they are likely generated directly from late BFUe or pre-CFUe with a $CD34^-/EP\text{-}1^+$ phenotype. Several pieces of evidence favor this view: during the 6 days in non-Epo suspension cultures, CFUe, originally absent at day 0, never become more prevalent than either BFUe or globin(+) cells (Tables 2 and 4); this holds true under conditions that either increase (Fig 1) or curtail (Table 2) BFUe output; double-labeling experiments with EP-1 and CD34 point to a subset of $CD34^-/EP\text{-}1^+$ cells that give rise to many late BFUe colonies at day 0 and generate more globin(+) cells by day 6 than any other subset (Table 5); again in this subset, both BFUe (mostly late) and globin(+) cells were higher than CFUe up to 6 days in suspension culture (Table 5). Whatever their stage of differentiation might be (pre-CFUe or CFUe-like), the day 6 globin(+) cells do not appear to mature when cultures are prolonged; they die retaining their immature state. This may be the main reason why this population, although generated regularly in small numbers (as small colonies) in standard clonal cultures, especially those with IL-3 or KL but no Epo

(Th. Papayannopoulou, unpublished data), is not usually recognized. Their presence is probably evanescent, and they die if Epo is not continuously present. Thus, although their generation in vitro (in the presence of IL-3 or KL) is Epo-independent, their further amplification and maturation under ordinary culture conditions is absolutely dependent on Epo. Whether erythroid cells at their earliest stages of differentiation express a different Epo receptor isoform, thus contributing to their apoptotic death, as it has been recently suggested,[52] remains to be seen.

Is the generation of globin(+) cells under our culture conditions only an in vitro phenomenon, or does it have any relevance to in vivo differentiation? Although our culture conditions contain rather high levels of powerful cytokines, we believe that this population is generated in vivo as well. Such cells usually escape detection as their numbers are very low and their only recognizable characteristic may be the presence of globin. The fact that rare globin(+) blast-like cells were seen in some of our preparations of circulating purified progenitors at day 0 favors the notion that this population does exist in vivo.

Recently, evidence was presented that KL increases the proportion of HbF in clonal cultures of human progenitors, both in serum-containing and serum-deprived cultures.[53] Our present observations in suspension cultures failed to show consistent differences in HbF proportion (either in γ-positive cells or in γ-mRNA compared with β-mRNA) in cultures with or without KL. On the other hand, a dominant negative effect on HbF of our serum-free medium was demonstrated in all combinations tested despite the immaturity of the tested population. Whether higher doses of KL than the ones used here would increase HbF in synergy with serum factors, as claimed,[53] requires further testing.

The mechanism(s) by which KL in synergy with IL-3 leads to accumulation of globin(+) cells is of extreme interest and requires further study. It is unclear from our data whether KL influences only the survival and/or proliferation of spontaneously generated globin(+) cells or, in addition, enhances their direct generation from BFUe. It is possible that, under the synergistic influence of KL, pivotal erythroid specific transcriptional factors, such as GATA-1,[54] are upregulated in BFUe progeny in the absence of Epo ligand/receptor induced interactions.[55] Indeed, in preliminary experiments we have observed such a GATA-1 upregulation (unpublished data) in IL-3 + KL-containing cultures. Upregulation of GATA-1 in non-Epo-containing cultures (also observed by Sposi et al[56]) may explain the early appearance of globin(+) cells in these cultures. However, it does not seem to be sufficient for continuing maturation and expression of a well-coordinated erythroid program. In this context, it is of interest that the phenotype of our globin(+) cells is reminiscent of the phenotype of many human[33] and murine (ME-26)[57] erythroleukemia cells and cell lines, which express significant levels of GATA-1 and globin, but relatively little or no heme and for the most part fail to mature. However, one difference between these leukemic cells and the normal cells studied here is that Epo can induce maturation in the normal cells, but it cannot do so in most leukemic cell lines.

We believe that our results provide new information of relevance to the understanding of early events in erythropoiesis, as they expand the target cell repertoire for KL to include cells (eg, at the CFUe level) thought to be influenced by Epo only. Any KL or anti–c-*kit* effect on survival and/or amplification of these cells would be masked when Epo is present at full doses (Table 2). This would also reconcile the previously reported absence of KL effect on CFUe, as it was tested in Epo-containing cultures.[38] In addition to their direct bearing on the physiology of erythropoiesis, our observations may provide an insight into the predominant effect of KL on in vivo erythropoiesis in W and Steel mutant mice. As implied by our in vitro data, and in concert with a previous publication,[58] KL in synergy with Epo may be critical for the amplification in vivo of later stages in erythropoiesis, beyond BFUe. This may explain the inability of W and Steel mutants, with low *kit* ligand/receptor function but normal Epo levels, to meet their anemic demands, met in normal mice by an expanded later progenitor/precursor pool of erythroid cells.

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REFERENCES

1. Metcalf D: Control of granulocytes and macrophages: Molecular, cellular, and clinical aspects. Science 254:529, 1991

2. Koury MJ: Programmed cell death (apoptosis) in hematopoiesis. Exp Hematol 20:391, 1992

3. Takeshita K, Benz EJ: Analysis of gene expression during hematopoiesis: Present and future applications. CRC Crit Rev Oncol Hematol 4:67, 1985

4. Carrow CE, Hangoc G, Cooper SH, Williams DE, Broxmeyer HE: Mast cell growth factor (c-*kit* ligand) supports the growth of human multipotential progenitor cells with a high replating potential. Blood 78:2216, 1991

5. Bernstein ID, Andrews RG, Zsebo KM: Recombinant human stem cell factor enhances the formation of colonies by $CD34^+$ and $CD34^+lin^-$ cells, and the generation of colony-forming cell progeny from $CD34^+lin^-$ cells cultured with interleukin-3, granulocyte colony-stimulating factor, or granulocyte-macrophage colony-stimulating factor. Blood 77:2316, 1991

6. Dai CH, Krantz SB, Zsebo KM: Human burst-forming units-erythroid need direct interaction with stem cell factor for further development. Blood 78:2493, 1991

7. Muench MO, Schneider JG, Moore MAS: Interactions among colony-stimulating factors, IL-1β, IL-6, and kit-ligand in the regulation of primitive murine hematopoietic cells. Exp Hematol 20:339, 1992

8. Lu L, Leenhuis T, Srour EF, Yang Y-Ch: Human interleukin (IL)-9 specifically stimulates proliferation of $CD34^{+++}DR^+CD33^-$ erythroid progenitors in normal human bone marrow in the absence of serum. Exp Hematol 20:418, 1992

9. Xiao M, Leemhuis T, Broxmeyer HE, Lu L: Influence of combinations of cytokines on proliferation of isolated single cell-sorted human bone marrow hematopoietic progenitor cells in the absence and presence of serum. Exp Hematol 20:276, 1992

10. McAlister IB, Teepe M, Gillis S, Williams D: *Ex vivo* expansion of peripheral blood progenitors with recombinant cytokines. Exp Hematol 20:626, 1992

11. Brandt JE, Briddell RA, Srour EF, Leemhuis TB, Hoffman R: The role of c-*kit* ligand in the expansion of human hematopoietic progenitor cells. Blood 79:634, 1992

12. Bodine DM, Orlic D, Birkett NC, Seidel NE, Zsebo KM: Stem cell factor increases colony-forming unit-spleen number in vitro in synergy with interleukin-6, and in vivo Sl/Sld mice as a single factor. Blood 79:913, 1992

13. Metcalf D, Nicola NA: Direct proliferative actions of stem cell factor on murine bone marrow cells *in vitro:* Effects of combination with colony-stimulating factors. Proc Natl Acad Sci USA 88: 6239, 1991

14. Briddell RA, Broudy VC, Bruno E, Brandt JE, Srour EF, Hoffman R: Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to the c-*kit* receptor. Blood 79:3159, 1992

15. Migliaccio G, Migliaccio AR, Druzin ML, Giardina P-JV, Zsebo KM, Adamson JW: Long-term generation of colony-forming cells in liquid culture of CD34$^+$ cord blood cells in the presence of recombinant human stem cell factor. Blood 79:2620, 1992

16. Fibach E, Manor D, Oppenheim A, Rachmilewitz EA: Proliferation and maturation of human erythroid progenitors in liquid culture. Blood 73:100, 1989

17. Fibach E, Manor D, Treves A, Rachmilewitz EA: Growth of human normal erythroid progenitors in liquid culture: A comparison with colony growth in semisolid culture. Int J Cell Cloning 9:57, 1991

18. Sawada K, Krantz SB, Dai CH, Sato N, Ieko M, Sakurama S, Yasukouchi T, Nakagawa S: Transitional change of colony stimulating factor requirements for erythroid progenitors. J Cell Physiol 149:1, 1991

19. Ottman OG, Abboud M, Welte K, Souza LM, Pelus LM: Stimulation of human hematopoietic progenitor cell proliferation and differentiation by recombinant human interleukin-3. Comparison and interactions with recombinant human granulocyte-macrophage and granulocyte colony stimulating factors. Exp Hematol 17:191, 1989

20. Saeland S, Caux C, Favre C, Aubry JP, Mannoni P, Pebusque MJ, Gentilhomme O, Otsuka T, Yokota T, Arai N, Arai K, Banchereau J, de Vries JE: Effects of recombinant interleukin-3 on CD34-enriched normal hematopoietic progenitors and on myeloblastic leukemia cells. Blood 72:1580, 1988

21. Serke S, Sauberlich S, Huhn D: A liquid culture method for the in vitro growth of hemopoietic progenitor cells from normal human adult peripheral blood allowing for analysis by multiparameter flow-cytometry. Eur J Haematol 46:85, 1991

22. Maciejewski J, Weber H, Neuhaus K, Waechter M, Elstner E, Seiler FR, Volk HD: Comparative analysis of the influences of IL-1, IL-3, and GM-CSF on the commitment of granulocyte-macrophage progenitors in vitro. Ann Hematol 63:242, 1991

23. Egeland T, Steen R, Quarsten H, Gaudernack G, Yang Y-C, Thorsby E: Myeloid differentiation of purified CD34$^+$ cells after stimulation with recombinant human granulocyte-macrophage colony-stimulating factor (CSF), granulocyte-CSF, monocyte-CSF, and IL-3. Blood 78:3192, 1991

24. Spivak J: The mechanism of action of erythropoietin. Int J Cell Cloning 4:139, 1986

25. Krantz SB: Erythropoietin. Blood 77:419, 1991

26. Fagg B: Is erythropoietin the only factor which regulates late erythroid differentiation? Nature 289:184, 1981

27. Goodman JW, Hall EA, Miller KL, Shinpock SG: Interleukin-3 promotes erythroid burst formation in "serum-free" cultures without detectable erythropoietin. Proc Natl Acad Sci USA 82:3291, 1985

28. Monette FC, Sigounas G: Growth of murine multipotent stem cells in a simple "serum-free" culture system: Role of interleukin-3, erythropoietin, and hemin. Exp Hematol 16:250, 1988

29. Mayeux P, Felix JM, Billat C, Jacquot R: Induction by hemin of proliferation and differentiation of progenitor erythroid cells responsible for erythropoietin. Exp Hematol 14:801, 1986

30. Correa PN, Axelrad AA: Production of erythropoietic bursts by progenitor cells from adult human peripheral blood in an improved serum-free medium: Role of insulin-like growth factor 1. Blood 78: 2823, 1991

31. Broudy VC, Lin N, Zsebo KM, Birkett NC, Smith KA, Bernstein ID, Papayannopoulou Th: Isolation and characterization of a monoclonal antibody that recognizes the human c-*kit* receptor. Blood 79:338, 1992

32. Yokochi T, Brice M, Rabinovitch PS, Papayannopoulou Th, Stamatoyannopoulos G: Monoclonal antibodies detecting antigenic determinants with restricted expression on erythroid cells: From the erythroid committed progenitor level to the mature erythroblast. Blood 63:1376, 1984

33. Papayannopoulou Th, Nakamoto B, Kurachi S, Tweedale M, Messner H: Surface antigenic profile and globin phenotype of two new human erythroleukemia cell lines: Characterization and interpretations. Blood 72:1029, 1988

34. Constantoulakis P, Nakamoto B, Papayannopoulou Th, Stamatoyannopoulos G: Fetal calf serum contains activities that induce fetal hemoglobin in adult erythroid cell cultures. Blood 69:1862, 1990

35. Orkin SH, Harosi RI, Leder P: Differentiation in erythroleukemic cells and their somatic hybrids. Proc Natl Acad Sci USA 72: 98, 1975

36. McLeod DL, Shreeve MM, Axelrad AA: Improved plasma culture system for production of erythrocytic colonies in vitro: Quantitative assay method for CFUe. Blood 44:517, 1974

37. Cooper KD, Kang K, Bush TL, Hanifin JM: Direct depletion and purification of monoclonal antibody defined cells from unfractionated human mononuclear leukocytes using antibody coated polystyrene petri dishes. J Clin Lab Immunol 13:97, 1984

38. Papayannopoulou Th, Brice M, Broudy VC, Zsebo KM: Isolation of c-*kit* receptor-expressing cells from bone marrow, peripheral blood, and fetal liver: Functional properties and composite antigenic profile. Blood 78:1403, 1991

39. Ema H, Suda T, Miura Y, Nakauchi H: Colony formation of clone-sorted human hematopoietic progenitors. Blood 75:1941, 1990

40. Ratajczak M, Luger SM, De Riel K, Abrahm J, Calabretta B, Gewirtz AM: Role of the *c-kit* protooncogene in normal and malignant human hematopoiesis. Proc Natl Acad Sci USA 89:1710, 1992

41. Catlett JP, Leftwich JA, Westin EH, Grant S, Huff TF: c-*kit* expression by CD34$^+$ bone marrow progenitors and inhibition of response to recombinant human interleukin-3 following exposure to c-*kit* antisense oligonucleotides. Blood 78:3186, 1991

42. Fujimori Y, Ogawa M, Clark SC, Dover DJ: Serum-free culture of enriched hematopoietic progenitors reflects physiologic levels of fetal hemoglobin synthesis. Blood 75:1718, 1990

43. Migliaccio AR, Migliaccio G, Brice M, Constantoulakis P, Stamatoyannopoulos G, Papayannopoulou Th: Influence of recombinant hematopoietins and of fetal bovine serum on the globin synthetic pattern of human BFU-e. Blood 76:1150, 1990

44. Hermine O, Beru N, Pech N, Goldwasser E: An autocrine role of erythropoietin in mouse hematopoietic cell differentiation. Blood 78:2253, 1991

45. Prchal JF, Axelrad AA: Bone marrow responses in polycythemia vera. N Engl J Med 290:1382, 1974

46. Eridani S, Pearson TC, Sawyer B, Batten E, Wetherley-Mein G: Erythroid colony formation in primary proliferative polycythemia, idiopathic erythrocytosis and secondary polycythemia: Sensitivity to erythropoietic stimulating factors. Clin Lab Haematol 56:157, 1983

47. Mladenovic J, Adamson JW: Characteristics of circulating erythroid colony-forming cells in normal and polycythemic man. Br J Haematol 51:337, 1982

48. Lacombe C, Casadevall N, Varet B: Polycythemia vera: *In vivo* studies of circulating erythroid progenitors. Br J Haematol 44: 189, 1980

49. Pennathur-Das R, Alpen E, Vichinsky E, Garcia J, Lubin B: Evidence for the presence of CFUe with increased sensitivity to erythropoietin in sickle cell anemia. Blood 63:1168, 1984

50. Isoyama K, Baliga S, Shah A, Mankad VN: Increased response of erythroid progenitors to interleukin-3 in sickle cell anemia: CFUe-like behavior of circulating erythroid progenitors in liquid culture. Am J Hematol 38:101, 1991

51. Mankad VN, Moore RB, McRoyan D, Zukerman K: Erythrocytosis associated with spontaneous erythroid colony formation and idiopathic hypererythropoietinemia. J Pediatr 111:743, 1987

52. Nakamura Y, Komatsu N, Nakauchi H: A truncated erythropoietin receptor that fails to prevent programmed cell death of erythroid cells. Science 257:1138, 1992

53. Miller BA, Perrine SP, Bernstein A, Lyman SD, Williams DE, Bell LL, Olivieri NF: Influence of Steel factor on hemoglobin synthesis in sickle cell disease. Blood 79:1861, 1992

54. Tsai S-F, Martin DIK, Zon LI, D'Andrea AD, Wong GG, Orkin SH: Cloning of cDNA for the major DNA binding protein of the erythroid lineage through expression in mammalian cells. Nature 339:446, 1989

55. Chiba T, Ikawa Y, Todokoro K: GATA-1 transactivates erythropoietin receptor gene, and erythropoietin receptor-mediated signals enhance GATA-1 gene expression. Nucleic Acids Res 19: 3843, 1991

56. Sposi NM, Zon LI, Care A, Valtieri M, Testa U, Gabbianelli M, Mariani G, Bottero L, Mather C, Orkin SH, Peschle C: Cell cycle-dependent initiation and lineage-dependent abrogation of GATA-1 expression in pure differentiating hematopoietic progenitors. Proc Natl Acad Sci USA 89:6353, 1992

57. Aurigemma RE, Blair DG, Ruscetti SK: Transactivation of erythroid transcription factor GATA-1 by a myb-ets-containing retrovirus. J Virol 66:3056, 1992

58. Ohneda O, Yanai N, Obinata M: Combined action of c-*kit* and erythropoietin on erythroid progenitor cells. Development 114: 245, 1992

Journal of Cellular Biochemistry 21:151–159 (1983)
Evolution of Hormone-Receptor Systems 279–287

CSF-1—A Mononuclear Phagocyte Lineage-Specific Hemopoietic Growth Factor

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INTRODUCTION

All of the mature blood cell types of the mouse are derived from a single, pluripotent, hemopoietic stem cell [reviewed in 1]. These stem cells represent only a very small fraction (~0.1%) of the cells of the hemopoietic organs (yolk sac, fetal liver, bone marrow, and spleen). However, each stem cell has the potential to give rise to thousands of mature blood cells by a process of proliferation and differentiation. Owing to the relatively short life-span of blood cells, this process must occur continuously in order to maintain mature blood cell levels. A group of circulating hemopoietic growth factors are involved in regulating these events. They include erythropoientin (erythrocytic lineage) and the colony-stimulating factors (granulocytic and mononuclear phagocytic lineages).

The term "colony-stimulating factor" (CSF) embraces the group of growth factors that stimulate hemopoietic precursor cells to form clones containing granulocytes and/or macrophages [2,3]. At least four subclasses of CSF may be discerned by their different physical properties and their preferential stimulation of neutrophil, eosinophil, neutrophil-macrophage, or macrophage colony formation [reviewed in 4]. Two CSFs stimulate the proliferation of mononuclear phagocytes directly—CSF-1 and CSF_{GM}. CSF-1 [5,6] has been clearly discriminated from the other CSFs by its detection in subclass-specific radioimmuno- and radioreceptor assays [4,6–8]. It is lineage-specific, stimulating the survival, proliferation, and differentiation of mononuclear phagocytes and their precursors [4,9,10]. The macrophage growth factor from L-cell-conditioned medium described by Virolainen and Defendi [11] has been shown to be CSF-1 [12]. CSF_{GM} is a glycoprotein of M_r ~23,000 which stimulates the formation of colonies containing both neutrophilic granulocytes and macrophages and the proliferation of primitive precursor cells of the erythroid lineage [13,14]. It is

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significantly less effective than CSF-1 in generating macrophages in bone marrow cultures, and its actions on mononuclear phagocytes are not mediated by the CSF-1 receptor [8]. Studies with CSF_{GM} [reviewed in 4,14] have been limited by an inability to raise antibodies against it and to radioiodinate it with retention of biological activity.

This review summarizes our studies on the characterization and mechanism of action of CSF-1, and reports briefly on recent studies in which the CSF-1 receptor interaction has been used in the identification and assay of new hemopoietic growth factors.

ASSAY, PURIFICATION, AND PROPERTIES OF CSF-1

The bioassay for the CSFs is based on the CSF-dependent stimulation of bone marrow cells to form colonies of granulocytes and/or macrophages in semisolid agar [3] or methyl cellulose [2] culture media. Standardization and quantitation of this colony assay for CSF-1 have been reviewed elsewhere [4]. A limitation of the colony assay is that it cannot be used to specifically assay CSF-1 in preparations that contain the other CSF subclasses. However, measurement of the CSF-1 concentration in such preparations can be determined by CSF-1-specific competitive binding assays that are based on the ability of CSF-1 to compete for the binding of ^{125}I-CSF-1 to either anti-CSF-1 antibody or the CSF-1 receptor. Murine [7] and human [6] CSF-1 radioimmunoassays are relatively species-specific. However, the murine radioreceptor assay [8] can be used to detect either murine or human CSF-1. Both radioimmuno- and radioreceptor assays appear to detect only biologically active CSF-1 [15]. All CSF-1 assays (including the colony assay) can be standardized against stable preparations to which values in units have been ascribed [reviewed in 4]. One unit of pure CSF-1 contains ~6 pg of protein and gives rise to 1–10 colonies per 7.5 × 10^4 bone marrow cells [5,15].

CSF-1 has been purified from the medium conditioned by serum-free cultures of murine L cells [5,15], human MIA Pa Ca-2 cells [16], and human urine [6]. The L cell CSF-1 has been shown to be pure by comigration of protein and activity during polyacrylamide gel electrophoresis (PAGE) in the presence and absence of sodium dodecyl sulphate (SDS), during isoelectric focusing [5], and during SDS-PAGE following enzymatic removal of >85% of the CSF-1 carbohydrate moieties [17]. In addition, formation of the purified protein-antibody complex and the neutralization of its biological activity occur at the same concentration of antibody [5]. CSF-1 represents ~0.1% of the total protein of serum-free conditioned medium, which is the most appropriate starting material for its purification. Thus only small amounts of purified material (~200 μg) may be obtained from large volumes (~10 liters) of medium. This problem has been a major limiting factor in molecular studies of CSF-1 and its mechanism of action.

CSF-1 from both human and murine sources is an acidic glycoprotein of M_r ~45,000–~76,000 [5,6,17]. A schematic representation of the CSF-1 molecule is shown in Figure 1. Reduction, even in the absence of dissociating agents, destroys the biological activity and halves the M_r of all CSF-1 preparations, reflecting the existence of two similar subunits in the molecule. Studies with L cell CSF-1 indicate that these subunits are of similar charge [5]. The nature and extent of CSF-1 glycosylation has been investigated utilizing endoglycosidase treatment. Irrespective of the M_r of the reduced and alkylated CSF-1 (24,000–33,000), exhaustive treatment

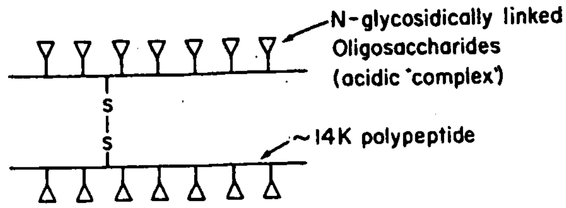

Fig. 1. Provisional structure of murine or human CSF-1 (schematic). The two polypeptide chains are very similar, if not identical. The number of oligosaccharides per molecule is variable and depends on the origin of the preparation. The number of disulfide bonds is unknown.

of reduced and alkylated CSF-1 with endo-β-N-acetylglucosaminidase-D, but not endo-β-N-acetylglucosaminidase-H, gives rise to a molecule of M_r ~16,500 of which the polypeptide chain portion accounts for ~14,000. Whereas the parent molecule binds concanavalin A, this product does not, indicating that, as intimated from earlier studies [18,19], heterogeneity in the saccharide component could explain the observed variation in M_r of CSF-1 [17]. These results also suggest that the two polypeptide chains in the dimeric CSF-1 molecule are very similar and possibly identical. Because of the specificity of endo-α-N-acetylglucosaminidase-D, it is concluded that the carbohydrate moieties are asparagine-linked, "complex-type" units. Examination of the effects of endo-α-N-acetyl-β-galactosaminidase treatment on the M_r of reduced and alkylated CSF-1 fails to provide evidence of the existence of 0-glycosidically linked oligosaccharides. Treatment of native CSF-1 with endo-β-N-acetylglucosaminidase-D removes carbohydrate almost as efficiently (>85%) as treatment of the reduced and alkylated subunits. Removal does not cause loss of antibody binding, receptor binding, or biological activity [17]. While the carbohydrate moiety does not appear to be necessary for the in vitro biological activity of CSF-1, it may be important for CSF-1 stability [19] and/or the relative resistance of CSF-1 to attack by proteolytic enzymes [20–22].

CELL TYPES EXHIBITING A PROLIFERATIVE RESPONSE TO CSF-1

Stimulation of proliferation by CSF-1 is restricted to cells of the mononuclear phagocytic series whether they be the colony-forming cells of bone marrow or spleen, blood monocytes, or tissue macrophages [reviewed in 4]. The maturation sequence of mononuclear phagocytic cell types and their properties are summarized in Table I. While CSF-1 stimulates the growth of both immature and relatively mature cells of the mononuclear phagocytic series, the immature cells have a higher proliferative capacity and a shorter doubling time than the more mature cells. Mononuclear phagocytes do not become "immortal" (ie, form continuous cell lines) if cultured with CSF-1, but instead have finite capacities for proliferation and eventually give rise to populations of nondividing cells [23]. Mononuclear phagocytes from many different tissues including bone marrow, blood, peritoneal cavity, pulmonary alveoli, and liver are all capable of exhibiting a proliferative response to purified CSF-1 [24]. However, while the proportion of freshly explanted monoblasts, promonocytes, or monocytes that are capable of extensive proliferation is >90%, the proportion of macrophages capable of extensive proliferation varies from ~1% (peritoneal macro-

TABLE I. Properties of Freshly Explanted Cells of the Mononuclear Phagocytic Lineage

	Property			
Cell type	% Cells capable of proliferation	Proliferative capacity of cells capable of proliferation	CSF-1 receptor	Glass adherent cells
CFU-C[a]		+++	+	–
↓				
Monoblast	>90	++	+	+
↓				
Promonocyte	>90	++	+	+
↓				
Monocyte	>90	++	+	+
↓				
Macrophage	1–50[b]	+	+	+

[a]Undifferentiated, nonadherent, colony-forming cell.
[b]Percent varies depending on source—eg, 50% of peritoneal exudate macrophages, 1% of resident peritoneal macrophages.

phages) to ~50% (peritoneal exudate macrophages). As this kind of variation among different macrophage populations is not observed for the CSF-induced release of plasminogen activator [25], proliferative senescence within the mononuclear phagocytic series may not necessarily be associated with a loss of the regulation of other functions by CSF-1.

STIMULATION OF CELL SURVIVAL AND DIFFERENTIATION BY CSF-1

In addition to its effects on proliferation, CSF-1 stimulates the survival and differentiation of mononuclear phagocytes. Bone marrow–derived macrophages, resident peritoneal macrophages, and peritoneal exudate macrophages will die if cultured in the presence of culture medium containing only 15% fetal calf serum [10]. Addition of CSF-1, at concentrations below those stimulating significant cell proliferation (~20 pM), will prevent cell death. The effect of CSF-1 on the differentiation of immature mononuclear phagocytes has been less well studied; nevertheless, it is clear that the nonadherent mononuclear phagocyte precursor cells (CFU-C) of bone marrow rapidly differentiate into adherent, proliferating macrophages if pure CSF-1 alone is added to serum-containing culture medium (Tushinski and Bartelmez, unpublished observations).

CSF-1 RECEPTORS AND THE NATURE AND DISTRIBUTION OF CSF-1 BINDING CELLS

Detailed analysis of the binding and uptake of a growth factor is important for the identification of the cells with which it interacts directly and for the elucidation of the biochemical mechanisms underlying its effects on target cells. CSF-1 can be radiolabeled with ^{125}I to high specific radioactivity (300,000 cpm/ng) without loss of biological or antibody binding activity [5,15]. Initial studies of the binding and uptake of murine ^{125}I-CSF-1 have been carried out on murine peritoneal exudate macrophages, 50% of which are capable of extensive proliferation in the presence of CSF-1

and >95% of which were shown to bind ^{125}I-CSF-1 specifically [26]. The binding (at 4°C) of ^{125}I-murine CSF-1 to these macrophages is of high affinity ($K_d \leqslant 10^{-13}$ M, Table II) and not competed for by other known CSF subclasses, growth factors, or hormones [26]. A variety of approaches, including thick-section light autoradiography, temperature jump, and pH 4 dissociation experiments, indicate that ^{125}I-CSF-1 binds a cell surface receptor at 4°C (Guilbert et al, in preparation).

The interaction of ^{125}I-CSF-1 with peritoneal exudate macrohages at 37°C is complex. ^{125}I-CSF-1 is destroyed by macrophages at 37°C by a process involving CSF-1 receptor-mediated internalization, significant intracellular accumulation, and subsequent intralysosomal degradation [17] (Guilbert et al, in preparation). The relationship between CSF-1 degradation and the proliferative response of cells is not clear at the present time.

Studies of the distribution, frequency, and morphology of murine binding cells [26,27] indicate that binding is restricted to cells of the mononuclear phagocytic system, and that the CSF-1 receptor is an excellent marker of mononuclear phagocytic cells, irrespective of their tissue of origin or state of differentiation (Table III). Furthermore, the occurrence of the receptor on continuous murine cell lines is restricted to macrophage or myelomonocytic cell lines [26].

TABLE II. Parameters of Murine ^{125}I-CSF-1 Binding to Murine Peritoneal Exudate Macrophages at 4°C, pH 7.35

Parameter	Value
On-rate constant (k_{on})	$\sim 10^{8}$ M^{-1} min^{-1}
Off-rate constant (k_{off})	$\leqslant 10^{-5}$ min^{-1}[a]
Dissociation constant (k_{off})/(k_{on})	$\leqslant 10^{-13}$ M

All data derived from kinetic analyses. Equilibrium methods could not be used owing to instability of the empty receptor sites relative to the occupied receptor sites [15].
[a]Possibly irreversible.

TABLE III. Distribution and Frequency of ^{125}I-CSF-1 Binding Cells in Murine Tissues

Cell type	Percent labeled cells	Average number of ^{125}I-CSF-1 molecules/labeled cell
Bone marrow	4.3	5,600
Spleen	2.4	9,600
Blood mononuclear	7.5	3,000
Peritoneal exudate	ND (98.0)	ND (73,000)
Peritoneal	17.0 (95.0)	10,000 (24,000)
Alveolar	11.8	16,000
Lymph node	0.4	8,000
Blood granulocyte	0	0
Thymus	0	0

Data obtained from autoradiographs and cell binding experiments on freshly explanted cells [27]. ND, not done. Figures in parentheses are from the adherent fractions of these cell populations that were cultured for 24 hr in the absence of CSF-1 prior to binding [15,26]. With the exception of the alveolar cells, the proportion of labeled cells in the cell population was not significantly different from the reported proportion of mononuclear phagocytes. In the case of the alveolar cells, the proportion of labeled cells was significantly lower than the reported proportion of mononuclear phagocytes, and macrophages could be seen that did not have grains associated with them.

MECHANISM OF CSF-1 ACTION

In order to study the cell biological and molecular aspects of the mechanism of CSF-1 action, it is desirable to work with homogeneous populations of target cells. The more immature cells of the mononuclear phagocytic series represent a very small proportion of the cells in the tissues in which they occur (bone marrow, spleen, blood; Table III). As it is difficult to purify large numbers of these cells, their effectiveness as a target cell population is limited. On the other hand, the more mature cells (macrophages) are easily isolated as relatively pure populations but exhibit heterogeneity in their proliferative responses (Table I) and are contaminated with CSF-1-producing cells of fibroblastoid morphology. The latter problems, however, can be overcome by culturing mouse bone marrow cells in the presence of CSF-1-containing preparations for 3 days and selecting from the nonadherent (or undifferentiated; Table I) fraction those cells that become adherent—ie, differentiate, during a subsequent 2-day culture period. These selected cells, termed "bone marrow-derived macrophages" (BMM) because of their macrophage morphology, represent a homogeneous population that responds to CSF-1 by extensive proliferation and >95% of which binds ^{125}I-CSF-1 [10].

The effects of CSF-1 on BMM morphology have been studied at the light-microscopic level. Relative to nonproliferating BMM, proliferating BMM are much enlarged and have a "foamy" appearance owing to the presence of numerous phase-lucent vacuoles. As these vacuoles do not stain with Oil Red O and do not possess cytochemically demonstrable acid phosphatase activity, they are probably pinocytic in origin. Proliferating cells also exhibit increased membrane ruffling and possess larger numbers of filopodia than do nonproliferating cells [10]. These latter changes appear within minutes after CSF-1 addition to nonproliferating cells (Tushinski et al, in preparation). The relationship between CSF-1-induced vacuolation, the intralysosomal destruction of CSF-1, and the proliferative response to CSF-1 is currently being studied in BMM populations.

Studies on the requirement of CSF-1 for BMM proliferation indicate that its presence during the G_1 phase of the cell cycle is both necessary and sufficient for entry of BMM into S phase and their progression through G_2 and M (Tushinski et al, in preparation). Removal of CSF-1 from exponentially growing BMM cultured in 15% fetal calf serum-containing medium decreases the rate of DNA synthesis by more than 100-fold. Addition of CSF-1 to these cells causes them to resume DNA synthesis within 10–12 hr, but has more immediate effects on protein metabolism. Within 2 hr after CSF-1 addition, the BMM protein synthetic rate is maximally stimulated and this, together with the CSF-1-induced decrease in the rate of intracellular protein degradation, leads to an accumulation of total cell protein, which is apparent in as little as 2 hr after stimulation. The increased protein synthetic rate is a linear function of the CSF-1 concentration, whereas the inhibition of protein degradative rate is an exponential function of the CSF-1 concentration, and inhibition of the protein degradative rate may be an integral part of the mechanism by which CSF-1 induces BMM survival [40]. Current studies of the mechanism of CSF-1 action on BMM are directed toward the analysis of very rapid events, including ion transport and protein phosphorylation, which may be induced by the growth factor.

Because of the limited amount of pure CSF-1 available for whole animal work, almost all studies of the mechanism of CSF-1 action have been carried out in vitro.

However, measurement of CSF levels and colony-forming cells in a variety of experimental and clinical situations is consistent with an involvement of the CSFs as a group in the physiological regulation of granulocyte and macrophage production [reviewed in 28]. Other in vitro studies indicate that CSF-1 stimulates the release of plasminogen activator [25,29], prostaglandins [30], and interleukin-1 [31] by macrophages. It is apparent from these observations and from its separate effects on cell morphology, protein synthetic, and protein degradative rates, that CSF-1 regulates a pleiotropic response by mononuclear phagocytes.

GROWTH FACTORS STIMULATING MONONUCLEAR PHAGOCYTE PROGENITOR CELL PRODUCTION

It is clear that CSF-1 plays an important role in the generation of mononuclear phagocytes from CFU-C and in the regulation of the activities of mononuclear phagocytes in general. However, the question remains as to how the CFU-C themselves are derived from pluripotent, hemopoietic stem cells and whether hemopoietic growth factors are involved in that process. Bradley and colleagues [32,33] have developed an assay for a precursor cell of the CFU-C. They have termed this precursor the high-proliferative potential colony-forming cell (HPP-CFC). HPP-CFCs share many properties with hemopoietic stem cells and can be stimulated to form much larger colonies of macrophages than those formed by CFU-C in the presence of CSF-1. Formation of these larger colonies requires the presence of both CSF-1 and a factor which synergizes with CSF-1 [34] (Bartelmez et al, unpublished observations). In order to investigate the roles of the synergistic factor and other hemopoietic growth factors that might regulate the generation of CFU-C, we have developed assays which detect factors that stimulate an increase in the numbers of nonadherent ^{125}I-CSF-1 binding cells during short-term culture. Two growth factors have been resolved. Both increase the number of CSF-1-binding, nonadherent cells—one in the absence of CSF-1, while the other requires CSF-1 for its action. These factors ($M_r \sim 30{,}000$ and $M_r \sim 20{,}000$, respectively) act on more immature cells than those that respond to CSF-1 alone and appear to be distinct from CSF_{GM}. Current studies in this area are directed toward the purification and characterization of these new hemopoietic growth factors and analysis of their effects on the generation of precursor cells for other hemopoietic cell lineages.

SUMMARY AND CONCLUSIONS

CSF-1 is the only mononuclear phagocyte lineage-specific growth factor described to date. Despite its unusually high degree of glycosylation, it appears that a completely deglycosylated form might still retain in vitro biological activity. This conclusion raises the question of the function of the CSF-1 oligosaccharides. Since CSF-1 acts on macrophages that release proteases [35–37] and which are found with CSF-1 in areas of local inflammation, its substantial glycosylation may provide protection from extracellular degradation. It is interesting to note that without its carbohydrate, CSF-1 shares structure-activity relationships with the biologically ac-

tive subunit of nerve growth factor (β-NGF). β-NGF is a dimer composed of two noncovalently associated polypeptide chains of $M_r \sim 14{,}000$ with identical or completely overlapping sequences [38]. Determination of the amino acid sequence of the CSF-1 polypeptide chain(s) will resolve the question of whether CSF-1 is related to β-NGF, which belongs to the family of insulin-related growth factors [39].

As in the case of other growth factors and the polypeptide hormones, the biological effects of CSF-1 are mediated by a specific cell-surface receptor. These effects include the stimulation of target cell survival, proliferation, and differentiation as well as other morphological and functional changes. Studies on the mechanism(s) by which the CSF-1 receptor system leads to the pleiotropic effects of CSF-1 on mononuclear phagocytes will be facilitated by the recent development of methods for obtaining homogeneous populations of target cells (BMM). However, the restricted occurrence of the CSF-1 receptor among cells of the mononuclear phagocytic series makes it an excellent marker of this lineage, and has already led to the delineation of two new growth factors that may be involved in directing hemopoietic cell proliferation and differentiation at the level of the hemopoietic stem cell. These latter studies may help elucidate the basic mechanisms by which several growth factors interact in the generation of mature cells from immature precursor cells in proliferating tissues.

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REFERENCES

1. Till JE, McCulloch EA: BBA 605:43, 1980.
2. Pluznik DH, Sachs L: Exp Cell Res 43:533, 1966.
3. Bradley TR, Metcalf D: Aust J Exp Biol Med Sci 44:287, 1966.
4. Stanley ER: In Stewart WE II, Hadden JW (eds): "The Lymphokines." New Jersey: Humana Press, 1981, pp 102–132.
5. Stanley ER, Heard PM: J Biol Chem 252:4305, 1977.
6. Das SK, Stanley ER, Guilbert LJ, Forman LW: Blood 58:630, 1981.
7. Stanley ER: Proc Natl Acad Sci USA 76:2969, 1979; see also erratum ibid 76:5411, 1979.
8. Das SK, Stanley ER, Guilbert LJ, Forman LW: J Cell Physiol 104:359, 1980.
9. Stanley ER, Guilbert LJ: In Van Furth R (ed): "Mononuclear Phagocytes Functional Aspects." Boston, The Hague, London: Martinus Nijhoff, 1980, Part I, pp 417–433.
10. Tushinski RJ, Oliver IT, Guilbert LJ, Tynan PW, Warner JR, Stanley ER: Cell 28:71, 1982.
11. Virolainen M, Defendi V: In Defendi V, Stoker M (eds): "Growth Relating Substances for Animal Cells in Culture." Philadelphia: Wistar Institute Press, 1967, Symposium Monograph 7, pp 67–85.
12. Stanley ER, Cifone M, Heard PM, Defendi V: J Exp Med 143:631, 1976.
13. Burgess AW, Camakaris J, Metcalf D: J Biol Chem 252:1998, 1977.
14. Burgess AW, Metcalf D: Blood 56:947, 1980.
15. Stanley ER, Guilbert LJ: J Immunol Meth 42:253, 1981.
16. Wu M-C, Cini JK, Yunis AA: J Biol Chem 254:6226, 1979.
17. Das SK, Stanley ER: J Biol Chem 257:13679–13684, 1982.

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:rsey: Humana Press,

5411, 1979.

Functional Aspects."

28:71, 1982.
ıbstances for Animal
ograph 7, pp 67–85.

18. Ayusawa D, Isaka I, Seno T, Tomida M, Yamamoto Y, Hozumi M, Takatsuki A, Tamura G: Biochem Biophys Res Commun 90:783, 1979.
19. Tsuneoka K, Shikita M, Takatsuki A, Tamura G: J Biochem (Tokyo) 90:611, 1981.
20. Stanley ER, Metcalf D: Aust J Exp Biol Med Sci 47:467, 1969.
21. Stanley ER, Metcalf D: Proc Soc Exp Biol Med 137:1029, 1971.
22. Stanley ER, Bradley TR, Sumner MA: J Cell Physiol 78:301, 1971.
23. Van der Zeijst BAM, Stewart CC, Schlesinger S: J Exp Med 147:1253, 1978.
24. Stanley ER, Chen D-M, Lin H-S: Nature 274:168, 1978.
25. Hamilton JA, Stanley ER, Burgess AW, Shadduck RK: J Cell Physiol 103:435, 1980.
26. Guilbert LJ, Stanley ER: J Cell Biol 85:153, 1980.
27. Byrne PV, Guilbert LJ, Stanley ER: J Cell Biol 91:848, 1981.
28. Metcalf D: In "Hemopoietic Colonies: In Vitro Cloning and Leukemic Cells". Berlin: Springer-Verlag, 1977.
29. Lin HS, Gordon S: J Exp Med 150:231, 1979.
30. Kurland JI, Pelus LM, Ralph P, Bockman RS, Moore MAS: Proc Natl Acad Sci USA 76:2326, 1979.
31. Moore RN, Hoffeld JR, Farrar JJ, Mergenhagen SE, Oppenheim JJ, Shadduck RK: In Pick E (ed): "The Lymphokines." New York: Academic Press, 1981, Vol 3, pp 119–130.
32. Bradley TR, Hodgson GS: Blood 54:1446, 1979.
33. Bradley TR, Hodgson GS, Bertoncello I: In Baum SG, Ledney GD, Bekkum VDW (eds): "Experimental Hematology Today." Basel: Karger, 1980, pp 285–297.
34. Kreigler AB, Bradley TR, Januszewicz E, Hodgson GS, Elms EF: Blood 60:503, 1982.
35. Unkeless J, Gordon S, Reich E: J Exp Med 139:834, 1974.
36. Wahl LM, Wahl SM, Mergenhagen SE, Martin GR: Proc Natl Acad Sci USA 71:3598, 1974.
37. Werb Z, Gordon S: J Exp Med 142:361, 1975.
38. Server AC, Shooter EM: Adv Protein Chem 31:339, 1977.
39. Bradshaw RA, Niall HD: Trends Biochem Sci 3:274, 1978.
40. Tushinski RJ, Stanley ER: J Cell Physiol (in press).